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Cadmium-induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition

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Summary

Exposure to environmentally prevalent heavy metals such as cadmium can have detrimental effects on a variety of commercially and ecologically important species such as oysters. Since Cd^{2+} is known to induce apoptosis in immune cells of vertebrates, we have investigated the effects of this metal on isolated oyster hemocytes, the main cellular immune defense in mollusks. Enhanced apoptosis of these cells could conceivably create immunosuppressed conditions in these organisms and result in reduced disease resistance and increased opportunistic infection, resulting in decline of their populations. Cd^{2+} exposure induced apoptosis in oyster hemocytes in a dose-dependent manner in the range of 10–100 μmol l^{-1}, as indicated by the translocation of phosphatidylserine to the outer leaflet of the plasma membrane. At higher concentrations (200–1000 μmol l^{-1}), there was no further increase in apoptosis but a significant increase in the level of necrosis. In stark contrast to vertebrate immune cells, there was no decrease in the mitochondrial membrane potential or activation of caspases in response to Cd^{2+} in the apoptotic range. Surprisingly, Cd^{2+} exposure in this range did cause a significant decrease in intracellular ATP levels, indicating a severe disturbance of energy metabolism. Similarly, Cd^{2+} exposure of isolated mitochondria resulted in partial uncoupling of mitochondria but no difference in mitochondrial membrane potential. The results demonstrate that the important environmental pollutant Cd^{2+} induces apoptosis in oyster immune cells and does so through a mitochondria/caspase-independent pathway, suggesting that a novel, perhaps ancient, apoptotic pathway is active in these cells. Furthermore, it appears that the observed decrease in ATP production during apoptosis is not due to the loss of the mitochondrial proton-motive force but is more likely to be due to inhibition of the F_0/F_1-ATPase and/or mitochondrial ADP/ATP or substrate transport.

Key words: cadmium, heavy metal, apoptosis, necrosis, oyster, hemocyte, *Crassostrea virginica*.

Introduction

Innate immunity of invertebrates, including bivalves, consists of humoral components of hemolymph (agglutinins, lysosomal enzymes, opsonizing molecules, antimicrobial peptides) and cellular defense performed by phagocytes (hemocytes or blood cells) and is considered to be an evolutionary ancient defense system (reviewed in Canesi et al., 2002). Hemocytes represent the first line of internal defense against parasites, pathogens and non-self materials in mollusks and play an important role in disease prevention. Their function is regulated by internal factors (such as hormones and humoral factors present in hemolymph) and is also highly sensitive to external disturbances, such as temperature, salinity and particularly chemical pollution of the environment (see Livingstone and Pipe, 1992; Fournier et al., 2002; Lacoste et al., 2002; Sauve et al., 2002a,b and references therein). Environmental pollutants can affect many hemocyte functions such as phagocytosis (Brousseau et al., 2000; Auffret et al., 2002; Sauve et al., 2002b), aggregation ability (crucial for encapsulation of foreign particles; Auffret and Oubella, 1997) and the ability to produce cytotoxic reactive oxygen species (ROS) used for bactericidal protection (Roesijadi et al., 1997; Auffret et al., 2002). Environmental pollution can also have significant effects on homeostasis of hemocyte cell number. For example, pollutants have been implicated in the increased levels of neoplastic disorders in bivalve populations from contaminated sites (Gardner et al., 1991; Livingstone and Pipe, 1992). On the other hand, some pollutants, especially heavy metals, are known to induce apoptosis and necrosis in invertebrates and vertebrates and may thus tip the balance of cellular homeostasis towards an increased cellular mortality (Li et al., 2000; Pourahmad and O'Brien, 2000; Robertson and Orrenius, 2000; Brousseau et al., 2000; Mičič et al., 2001; De

La Fuente et al., 2002; Sauve et al., 2002a,b; Sung et al., 2003). Enhanced cell death in the hemocyte population has the potential to generate an immunosuppressed organism with reduced capacity to resist pathological insults and opportunistic infections. Currently, nothing is known about the mechanisms of chemically induced apoptosis and necrosis in bivalve hemocytes, although pollutant-induced apoptosis has been studied in vertebrates (Fujimaki et al., 2000; Li et al., 2000, 2003; Pourahmad and O'Brien, 2000; Robertson and Orrenius, 2000; De La Fuente et al., 2002; Wätjen et al., 2002; Aydin et al., 2003; Shih et al., 2004).

Cadmium is an important environmental pollutant that is released into the environment as a result of human activities as well as natural processes such as leaching from cadmium-rich soils and rocks, volcanic activity or diatom deposition in marine sediment (GESAMP, 1987; Roesijadi, 1996; Frew et al., 1997). Populations of the eastern oyster, *Crassostrea virginica*, are exposed to varied cadmium level in estuaries and coastal areas. Like all marine bivalves, oysters have an ability to concentrate metals in soft tissues, accumulating large loads of heavy metals from water solutions and metal-contaminated sediments (reviewed in Roesijadi, 1996; Crompton, 1997). Resistance to the toxic effects of these pollutants is provided primarily by binding to metallothioneins and deposition of insoluble metal-containing granules (Roesijadi, 1996). However, these detoxification mechanisms are imperfect, and 10–80% of the total heavy metal pool in the cytosol of bivalves is bound to other proteins and low-molecular-mass compounds depending on the species and the regime of exposure to heavy metals (Giguere et al., 2003). These 'excess' metal ions have the potential to exert toxic effects, including cellular toxicity, on bivalves.

In vertebrates, it has been shown that cadmium exposure results in apoptosis, or programmed cell death, in a variety of cell types (Li et al., 2000; Pourahmad and O'Brien, 2000; Robertson and Orrenius, 2000; De La Fuente et al., 2002; Wätjen et al., 2002; Aydi et al., 2003; Shih et al., 2004). In response to this metal, cells activate the classical intrinsic death pathway, in which mitochondria have a central role. In this pathway, the mitochondrial inner membrane undergoes a permeability transition (also known as the MPT), resulting in a dramatic increase in permeability caused by an apparent opening of a channel known as the mitochondrial permeability transition pore (PTP) (reviewed in Leist and Nicotera, 1997; Mignotte and Vayssiere, 1998; Hüttenbrenner et al., 2003). Opening of this pore causes a decrease in the potential across the mitochondrial membrane ($\Delta\Psi_m$) and the release of cytochrome *c* (cyt *c*) into the cytoplasm. Cyt *c* interacts with apaf-1 and procaspase-9 to form a complex known as the apoptosome, which in turn activates effector caspases such as caspase-3. Activation of caspases eventually leads to degradation of the cell's DNA and to the cascade of other intracellular reactions that culminate in cell death by apoptosis. In vertebrates, the MPT is generally considered to be an early universal event in heavy-metal-induced apoptosis (Leist and Nicotera, 1997; Mignotte and Vayssiere, 1998; Hüttenbrenner et al., 2003).

Currently, there is very limited knowledge about the effects of cadmium on apoptosis in any cell types within the bivalves. In the mussel *Mytilus edulis*, short-term exposure to low non-toxic cadmium levels (1.8 μmol l^{-1}) was shown to decrease susceptibility of isolated gill cells to hydrogen peroxide-induced apoptosis, possibly due to induction of metallothioneins or other antioxidant molecules (Pruski and Dixon, 2002). Exposure to elevated levels of heavy metals such as cadmium, zinc, silver and mercury was shown to decrease viability of bivalve hemocytes (Brousseau et al., 2000; Sauve et al., 2002a,b), although the mechanisms of cell death were not elucidated. The molecular and cellular mechanisms of cadmium cytotoxicity in bivalves are not understood and nothing is known about the effects of cadmium on apoptosis of the hemocytes of bivalves. Understanding of the influence of this metal over this process on a cellular and molecular level is important because excessive apoptosis of hemocytes may have detrimental effects on the ability of oysters to fight infectious diseases (Sunila and LaBanca, 2003).

The aim of the present study was to investigate the effects of cadmium on hemocyte viability and apoptosis and to assess the role of cadmium-induced mitochondrial dysfunction in cell death in the eastern oyster, *Crassostrea virginica*. Our data show that cadmium induced apoptosis and, at higher levels, necrosis in isolated oyster hemocytes. The onset of apoptosis was associated with a 60–80% reduction of ATP levels in oyster hemocytes, whereas a much stronger depletion of ATP (by >90%) was found after exposure to cadmium in the necrosis-inducing range. Surprisingly, no MPT was apparent during Cd^{2+}-induced apoptosis in intact hemocytes or isolated oyster mitochondria, suggesting that this central apoptotic process is not involved in apoptosis in oysters as it is in vertebrates. Overall, our results contrast the earlier findings in vertebrates in which cadmium-induced apoptosis involved the MPT and activation of caspases and suggest that trace-metal-induced apoptosis may proceed by a different pathway in marine mollusks. This pathway may be novel or, more likely, an evolutionarily ancient route to death that is either no longer functional, is superceded or is masked by the feed-forward cascade activated by the MPT in vertebrates.

Materials and methods

Animal collection and maintenance

Adult oysters (*Crassostrea virginica* Gmelin; 80–120 mm shell length) were collected from Hewletts Creek near Wilmington, NC, USA. Water temperature at the time of collection was 14–16°C, and salinity varied between 24 and 30‰. The study site has very low background concentrations of heavy metals and organic pollutants (Mallin et al., 1999), with average cadmium concentrations of 0.02 mg kg^{-1} of sediment. Animals were transported on ice to the University of North Carolina at Charlotte within 8 h of collection and placed in recirculated aquaria with artificial seawater (Instant Ocean®; Kent Marine, Acworth, GA, USA) at 16±1°C and

650±20 mOsm, which was close to the environmental temperature and salinity at the time of collection. Animals were acclimated in the laboratory for 2 weeks prior to experimentation. During the acclimation period, oysters were fed on alternate days with a commercial algal blend (0.5 ml l^{-1}) containing *Nannochloropsis*, *Tetraselmis* and *Isochrysis* spp. ranging in size from 2 to 15 μm (PhytoPlex®; Kent Marine). No mortality was detected during the preliminary acclimation period.

Primary culture and cadmium exposure of oyster hemocytes

Oyster shells were surface-cleaned with ethanol and notched along the ventral edge enough to insert a needle. Hemolymph (1.5–2 ml) was extracted from an adductor muscle using a 21-gauge hypodermic needle and a syringe containing 0.5–1 ml of sterile hemocyte support medium (HSM) consisting of sterile seawater (SW; 650 mOsm) supplemented with 2 g l^{-1} glucose. Hemolymph from 3–5 oysters was pooled to obtain a total of 8–12×10^6 cells. Pooled samples were placed into wells of 6-well tissue culture plates to obtain a cell density of 1.5–2×10^6 cells per well and allowed to attach for 20–25 min at room temperature. Cell attachment was controlled under the inverted microscope. When the cells attached, hemolymph was removed, and 3 ml of HSM supplemented with 10 μg ml^{-1} gentamycin and 25 μg ml^{-1} amphotericin was added. $CdCl_2$ in 0.05% HNO_3 was added to different wells to obtain final nominal concentrations of 10–1000 μmol l^{-1} Cd^{2+}. Equal volumes of 0.05% HNO_3 were added to the control samples (0 μmol l^{-1} nominal Cd^{2+}). Hemocytes were incubated for 72 h at room temperature. Pilot studies have shown that incubation for at least 60 h is required to induce apoptosis in oyster hemocytes exposed to cadmium and that incubations in the range of 2–72 h have no significant effect on the viability of control hemocytes (data not shown).

Following cadmium exposure, hemocytes were harvested using 0.25% trypsin in modified Hanks balanced salt solution (Fisher Scientific, Suwanee, GA, USA) adjusted to 650 mOsm with sucrose, washed in sterile SW (650 mOsm) and used for further analyses.

Effects of cadmium on oyster cells

Annexin V–FITC assay

Following culture, cells were harvested, washed twice with SW, resuspended in 100 μl SW and stained for annexin V exposure on the plasma membrane using the annexin V–FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) and the manufacturer's recommendations. This staining allows the determination and quantification of apoptotic, necrotic and viable cells. Briefly, 100 μl of cell suspension (1.5–2.0×10^6 cells) was added to a 12×75 flow tube together with 5 μl each of the annexin V and propidium iodide (PI) solutions provided. Cells were incubated for 15 min at room temperature in the dark and then analyzed immediately by flow cytometry for fluorescence in the FL-1 (annexin V) and FL-2 (PI) channels.

Caspase assay

Caspase-3-like activity was measured using a fluorimetric assay as described elsewhere (Hughes et al., 1997; Jablonski et al., 2004). Briefly, hemocytes were resuspended in 100 μl of 10 mmol l^{-1} $MgCl_2$, 0.25% NP-40 and the extracts centrifuged at 100 000 ***g*** for 30 min to remove debris and particulate matter. Supernatants were harvested, combined with an equal volume of 40 mmol l^{-1} Hepes (pH 7.4), 20 mmol l^{-1} NaCl, 2 mmol l^{-1} EDTA, 20% glycerol and frozen at –70°C until assayed (less than 1 week). For positive apoptotic controls, rat thymocytes incubated for 18 h in the absence of the growth factor were used and extracted as described above. For analysis, cell extracts corresponding to 10–50 μg of protein, as measured by Bradford assay (Bradford, 1976), were incubated in 50 mmol l^{-1} Hepes (pH 7.5), 10 mmol l^{-1} dithiothreitol, 10% sucrose, 0.1% CHAPS with 200 μmol l^{-1} of the caspase-3 substrate DEVD-afc (Kamiya Biomedical Co., Seattle, WA, USA). Samples were incubated for 5 min at room temperature and their fluorescence at 505 nm measured (excitation at 400 nm) on a fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan). Samples were then incubated for an additional hour and fluorescence measured again. A standard curve of fluorescence *vs* free 7-amino-4-trifluromethylcoumarin (afc) was then used to calculate the specific activity of caspase-3-like enzymes per mg protein in each sample. For *in vitro* activation of caspase-3-like activity, extracts were preincubated with 10 μg ml^{-1} cyt *c* and 1 mmol l^{-1} dATP at room temperature for 1 h, and their caspase activity measured as described above.

Mitochondrial membrane potential

Control and cadmium-exposed hemocytes were washed twice in sterile SW, centrifuged for 10 min at 200 ***g*** and 4°C and resuspended in 1 ml of sterile SW to yield a final concentration of ~1×10^6 cells ml^{-1}. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using a method modified from Wong and Cortopassi (2002). Briefly, digitonin was added to the cell suspension to a final concentration of 10 μmol l^{-1} and incubated for 6 min on ice. Cells were then centrifuged for 10 min at 200 ***g*** and 4°C and washed twice with 5 ml sterile SW. Pilot studies have shown that this concentration of digitonin effectively permeabilizes cells without affecting the $\Delta\Psi_m$ (data not shown). The cells were resuspended in 1 ml of sterile SW and stained with 100 nmol l^{-1} tetramethylrhodamine methyl ester (TMRM; Molecular Probes, Eugene, OR, USA) for 30 min at room temperature in the dark before analysis by flow cytometry as the fluorescence in the FL-2 channel. After each analysis, the individual samples were treated with 25 μmol l^{-1} CCCP (carbonyl cyanide 3-chloropheryl hydrazone; Fisher Scientific) to collapse the $\Delta\Psi_m$, and the TMRM fluorescence was analyzed again.

ATP levels

ATP concentrations were measured in hemocytes exposed to 0–200 μmol l^{-1} Cd^{2+} for 72 h using CellTiter-Glo®

Luminescent Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Hemocytes were washed in sterile SW, lysed in a cell lysis buffer (CLB) containing 5 mmol l^{-1} Tris (pH 7.4), 20 mmol l^{-1} EDTA and 0.5% Triton X-100, and 50 μl of cell lysate was combined with an equal volume of CellTiter-Glo® Reagent, mixed on an orbital shaker for 2 min and incubated for 10 min at room temperature to stabilize luminescent signal. CLB without cells was prepared in the same way and used as a negative control. Luminescent signal was recorded using a Sirius luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) with a 10 s integration of the signal. In order to convert relative luminescence units (RLUs) into ATP concentrations, a calibration curve was constructed using 0.025–1 μmol l^{-1} ATP dissolved in CLB. The calibration curve was linear in the studied range of ATP concentrations. ATP levels in hemocyte lysates were determined by interpolation from the calibration curve. Although the initial number of cells per sample was similar in all replicates, cell mortality in cadmium-exposed hemocytes could result in variation in final cell numbers. To account for this variation, we measured protein concentrations in cell lysates using a modified Biuret assay (Bergmeyer, 1988) and expressed ATP concentrations as nmol ATP mg^{-1} protein.

Effects of cadmium on isolated mitochondria

Respiration

Hemocytes of oysters did not provide a sufficient amount of tissue for mitochondrial isolation; therefore, mitochondria were isolated from oyster gills using a standard method modified from Ballantyne and Moyes (1987). Isolation buffer had an osmolarity of 730 mOsm and consisted of 300 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 50 mmol l^{-1} NaCl, 8 mmol l^{-1} EGTA, 1% bovine serum albumin (BSA; essentially fatty acid free), 2 μg ml^{-1} of the protease inhibitor aprotinin and 30 mmol l^{-1} Hepes (pH 7.5). Previous studies have shown that isolation of oyster mitochondria in a slightly hyperosmotic medium maximizes coupling and yields superior quality mitochondria as compared with iso- or hypoosmotic media (Ballantyne and Moyes, 1987).

Gills of six or seven animals were removed, blotted dry and placed in 15 ml of ice-cold isolation medium. The tissue was homogenized with three passes (200 r.p.m.) of a Potter–Elvenhjem homogenizer using a loosely fitting Teflon pestle. The homogenate was centrifuged for 10 min at 2000 ***g*** and 2°C. The supernatant was collected, and the tissue pellet re-homogenized in 15 ml of ice-cold isolation buffer. The second homogenate was centrifuged at 2000 ***g***, and supernatants from the two centrifugations were pooled. The supernatant was then centrifuged at 10 500 ***g*** and 2°C for 12 min. The resulting mitochondrial pellet was washed twice with ice-cold EGTA-free isolation buffer to minimize cadmium binding and re-suspended in the ice-cold EGTA-free isolation buffer to give a mitochondrial protein content of 5–10 mg ml^{-1}.

Oxygen uptake by mitochondria was measured using Clarke-type oxygen electrodes (YSI, Yellow Springs, OH, USA) in 3 ml water-jacketed glass chambers equilibrated at 25°C. Three to five volumes of assay medium were mixed with one volume of isolation medium containing the mitochondria. The assay medium (AM) had an osmolarity of 650 mOsm and consisted of 150 mmol l^{-1} KCl, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} sucrose, 0.1% BSA (essentially fatty acid free), 2 μg ml^{-1} of aprotinin, 5 μmol l^{-1} myokinase inhibitor AP_5A and 30 mmol l^{-1} Hepes (pH 7.2). Different concentrations of $CdCl_2$ in the range of 5–50 μmol l^{-1} were added to the assay medium containing mitochondria and incubated for 5 min. Control mitochondria were incubated without cadmium addition. Addition of the highest concentration of cadmium used in this study (50 μmol l^{-1}) did not noticeably change the pH of the assay buffer (i.e. pH change was less than 0.01 units).

All assays were completed within 2–3 h of isolation of the mitochondria. Preliminary experiments have shown that there was no change in mitochondrial respiration or coupling during this period. Succinate was used as a substrate at saturating amounts (10–15 mmol l^{-1}) in the presence of 5 μmol l^{-1} rotenone. Maximal respiration rates of actively phosphorylating mitochondria (state 3) were achieved by addition of 50 μmol l^{-1} ADP, and state 4 respiration was determined in ADP-conditioned mitochondria as described by Chance and Williams (1955). State 4+ respiration was determined as oxygen consumption rate after addition of 2.5 μg ml^{-1} of the ATPase inhibitor oligomycin. State 4+ respiration in the presence of oligomycin is considered as a good upper-limit estimate of mitochondrial proton leak measured at high mitochondrial membrane potential (Brand et al., 1994; Kesseler and Brand, 1995; Abele et al., 2002). At the end of the assay, KCN (100 μmol l^{-1}) and salicylhydroxamic acid (SHAM; 200 μmol l^{-1}) were added to the mitochondria to inhibit mitochondrial respiration and to measure non-mitochondrial rate of oxygen consumption. SHAM was used in order to avoid the overestimation of non-mitochondrial respiration rate due to the presence of an alternative oxidase in bivalve mitochondria (Tschischka et al., 2000). In all cases, non-mitochondrial oxygen consumption rate was less than 1–5% of the state 3 respiration. Respiration rates in state 3, 4 and 4+ were corrected for non-mitochondrial respiration and oxygen electrode drift. Respiratory control ratio (RCR), which is a measure of mitochondrial coupling, was determined as the ratio of state 3 over state 4 respiration as described by Estabrook (1967). Protein concentrations in mitochondrial suspensions were measured using a modified Biuret method with 0.5% Triton X-100 added to solubilize the mitochondria (Bergmeyer, 1988). BSA was used as the standard. Protein content was measured for each batch of the isolation medium and subtracted from the total protein content of the mitochondrial suspension to determine the mitochondrial protein concentration. Oxygen solubility (β_{O_2}) for the assay medium at each experimental temperature was calculated as described in Johnston et al. (1994), and respiration rates were expressed as natom O min^{-1} mg^{-1} mitochondrial protein.

Mitochondrial swelling

Mitochondrial swelling was measured as described in Li et al. (2003). Mitochondria were isolated as described above, and 100 µl of mitochondrial suspension was added to 0.9 ml of standard AM containing 20 mmol l^{-1} succinate. Absorbance of the mitochondrial suspension was measured at 520 nm and 25°C using a UV/Vis Cary 50 spectrophotometer with a water-jacketed cuvette holder (Varian, Mulgrave, Victoria, Australia). After initial measurements, different concentrations of cadmium chloride were added to the cuvette. Mitochondria were incubated with cadmium for 20 min on ice, the cuvettes were then equilibrated for 5 min at 25°C, and changes in mitochondrial volume were monitored by measuring absorbance at 520 nm under constant stirring. A reduction in absorbance of a mitochondrial suspension indicates mitochondrial swelling and is a hallmark of the MPT (Li et al., 2003). As a positive control, mitochondria were incubated for 5 min in a hypoosmotic assay buffer (375 mOsm).

Mitochondrial membrane potential

$\Delta\Psi_m$ was determined as described in Scaduto and Grotyohann (1999). Briefly, mitochondria were isolated as described above, protein concentration was determined, and mitochondrial suspension was diluted to 2 mg ml^{-1} mitochondrial protein in the standard AM containing 0.5 µmol l^{-1} TMRM, 20 mmol l^{-1} succinate and 5 µmol l^{-1} rotenone. Mitochondria were incubated at 25°C for 10 min with 0–50 µmol l^{-1} Cd^{2+} until TMRM fluorescence stabilized. Concentrations of cadmium above 50 µmol l^{-1} were not used because preliminary experiments showed that they completely abolish respiration in isolated mitochondria (data not shown). Following cadmium incubations, TMRM fluorescence in mitochondrial suspension was measured under constant stirring at two excitation wavelengths (573 nm and 546 nm) and a single emission wavelength of 590 nm (excitation and emission slits 10 nm) using a fluorescence spectrophotometer (Hitachi Ltd). The ratio of the fluorescence at 590 nm when alternately excited by the two excitation wavelengths is a measure of the $\Delta\Psi_m$. It has been shown that the 573/546 ratio linearly increases with increasing $\Delta\Psi_m$ (Scaduto and Grotyohann, 1999). The mitochondrial uncoupler CCCP (50 µmol l^{-1}) was added to the mitochondrial suspension to collapse the membrane potential, and the 573/546 ratio was determined. All ratios were corrected for the background fluorescence of TMRM in the AM without mitochondria.

Statistics

Effects of cadmium concentrations were analyzed using split-plot repeated measures ANOVA after testing the assumptions of normality of data distribution and homogeneity of variances. Dunnet tests were used for *post-hoc* pairwise comparisons of sample means. Statistical analyses were performed using SAS 8.2 software (SAS Institute, Cary, NC, USA). Differences were considered significant if the probability for Type II error was less than 0.05.

Results

Exposure of isolated oyster hemocytes to 50 µmol l^{-1} Cd^{2+} induced morphological alterations characteristic of an apoptotic morphology in a subset of cells following 72 h of culture (Fig. 1). These changes included cell shrinkage, rounding up and extensive membrane blebbing. These morphological changes were also correlated with the distinctly apoptotic translocation of phosphatidylserine to the outer leaflet of the plasma membrane while retaining the integrity of this membrane. As shown in Fig. 2, the proportion of apoptotic cells (annexin V positive, PI negative – lower right quadrant of the dot plots in Fig. 2A) increased in a dose-dependent manner in the range of 10–100 µmol l^{-1} Cd^{2+} after 72 h incubation with cadmium (ANOVA; $F_{10,55}$=15.97, P<0.0001). Levels of apoptosis in oyster hemocytes were significantly above the control at Cd^{2+} levels of ⩾50 µmol l^{-1}. At even higher concentrations of Cd^{2+} (200–1000 µmol l^{-1}), no further increase in apoptosis was detected (Fig. 2B). While lower concentrations of Cd^{2+} led to negligible increases in necrosis (annexin V positive, PI positive – upper right quadrant of the

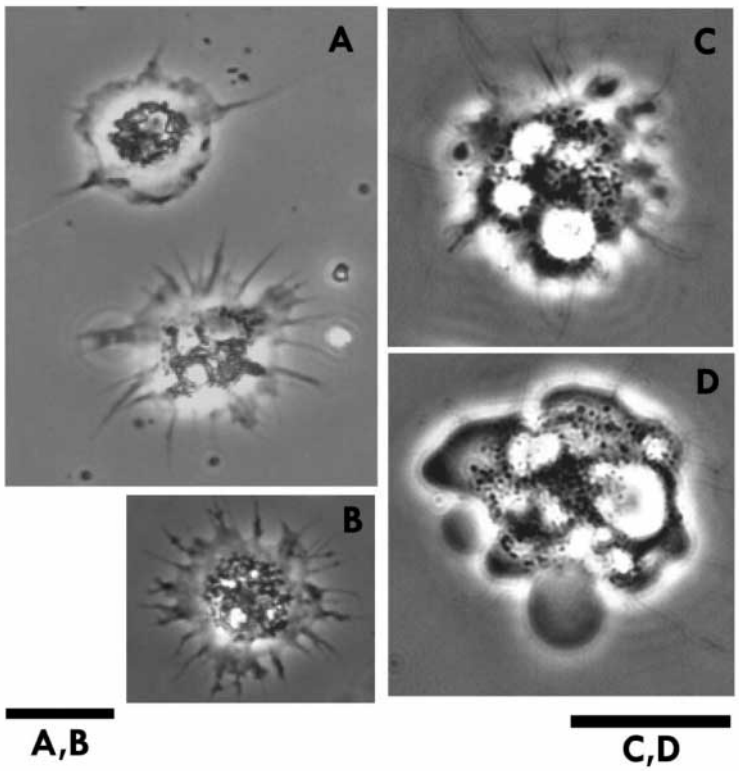

Fig. 1. Morphology of control oyster hemocytes (A,B) and presumptive apoptotic hemocytes exposed to 50 µmol l^{-1} of cadmium (C,D). Horizontal bars correspond to 10 µm. Note extensive blebbing in cadmium-exposed hemocytes (C,D).

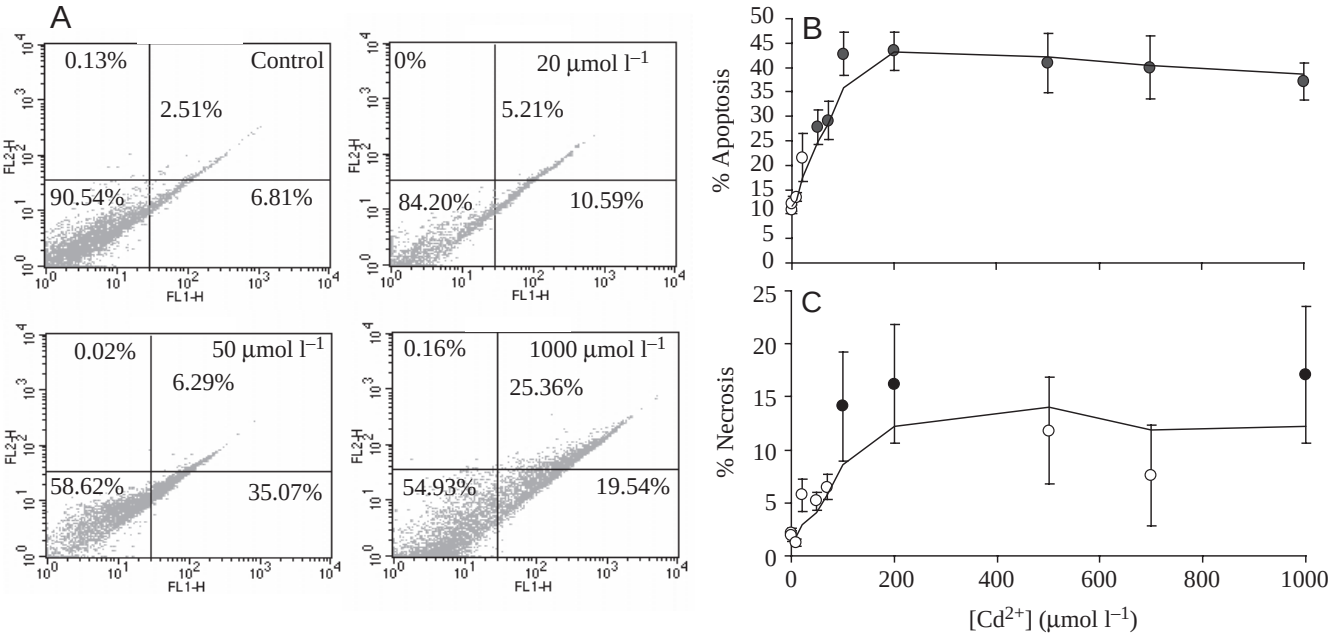

Fig. 2. Induction of apoptosis and necrosis by cadmium exposure in oyster hemocytes. (A) Representative dot plots for annexin V–FITC and propidium iodide (PI) staining in hemocytes exposed to vehicle (control) or varying concentrations of cadmium for 72 h. Live cells appear in the bottom left quadrant of the dot plot and have low FITC and PI fluorescence. Apoptotic cells in the bottom right quadrant are characterized by low PI fluorescence, indicating integrity of the plasma membrane, but high FITC fluorescence due to the translocation of phosphatidylserine into the outer leaflet of the plasma membrane. Necrotic cells have high PI and FITC fluorescence and are found in the upper right quadrant of the dot plot. (B,C) Quantitative graph of the data shown in A indicating the changes in the proportion of apoptotic (B) and necrotic (C) cells in the hemocyte population after 72 h of exposure to different cadmium concentrations. Vertical bars represent S.E.M. Filled circles correspond to the values that were significantly different from the respective control (0 μmol l^{-1} Cd^{2+}; $P<0.05$; $N=5–8$). The levels of apoptosis and necrosis in control hemocytes after 72 h of culture were not significantly different from those in freshly isolated oyster blood cells (12.0±1.70 and 0.9±0.34% of apoptosis and necrosis, respectively, $N=10$, $P>0.05$), indicating that culture conditions used in these experiments supported normal viability of oyster hemocytes.

dot plots in Fig. 2A), this increase was very apparent in the range from 50 to 200 μmol l^{-1} Cd^{2+}. Levels from 200 to 1000 μmol l^{-1} Cd^{2+} had no further effect on necrosis in this time period (Fig. 2C).

According to the mammalian model of cadmium-induced apoptosis, Cd^{2+} is known to induce apoptosis by triggering a change in mitochondrial membrane potential ($\Delta\Psi_m$) resulting in the release of cyt *c* and eventual activation of caspase-3. Thus, we next examined the $\Delta\Psi_m$ and caspase-3 activity in Cd^{2+}-treated oyster hemocytes. In contrast to our expectations, Cd^{2+} exposure did not facilitate a drop in $\Delta\Psi_m$ in oyster hemocytes (Fig. 3). On the contrary, exposure of hemocytes to 20–50 μmol l^{-1} Cd^{2+} resulted in a slight but significant hyperpolarization of mitochondrial membranes by 10–15% (ANOVA; $F_{3,19}=6.21$, $P=0.004$). At higher Cd^{2+} levels (200 μmol l^{-1}), the level of hyperpolarization tended to decrease but the $\Delta\Psi_m$ was still significantly higher than in the control cells. The mitochondrial uncoupler CCCP efficiently collapsed $\Delta\Psi_m$ to similar low levels independent of the cadmium treatment of oyster hemocytes. Control samples indicate that digitonin, which was used to permeabilize cell membranes and to facilitate penetration of TMRM and CCCP into the cells, had no significant effect on $\Delta\Psi_m$ in oyster hemocytes ($F_{1,19}=0.55$, $P=0.466$; Fig. 3, striped bar).

Analysis of activity of caspase-3-like enzymes, activated by the cyt *c* release during mitochondrial permeability transition, indicated that this activity was below the detection limit in freshly isolated oyster hemocytes and did not significantly increase in cells treated with vehicle nor the apoptotic (50 μmol l^{-1}) or necrotic (200 μmol l^{-1}) concentrations of cadmium ($F_{3,18}=0.86$, $P=0.48$; Fig. 4). Addition of dATP and cyt *c* to extracts of control hemocytes *in vitro* resulted in a dramatic increase in caspase-3-like activity up to levels close to those found in apoptotic rat thymocytes, which served as a positive control. This demonstrates that the inactive pro-form of this enzyme is present in the hemocyte extracts and suggests that, unlike the apoptosis induced in mammals, Cd^{2+} induction of cell death in oyster hemocytes does not activate this cascade.

Although there was no decrease in $\Delta\Psi_m$ due to cadmium exposure in oyster hemocytes, cellular ATP levels were decreased, indicating severe disturbance of energy status (Fig. 5). Cadmium exposure resulted in a significant depletion of ATP in oyster hemocytes (ANOVA; $F_{4,36}=4.63$, $P=0.004$), which was dose-dependent and manifest at cadmium levels (20 μmol l^{-1}) that did not significantly affect cell viability. Exposure to 20–50 μmol l^{-1} Cd^{2+}, which was in the range of apoptosis-inducing concentrations, was associated with a 60–88% decrease in ATP levels, and exposure to 200 μmol l^{-1}

Cd^{2+}, which induced significant levels of necrosis, resulted in a depletion of ATP levels by >90%.

Studies of the effect of cadmium on isolated oyster mitochondria *in vitro* corroborated our findings in intact hemocytes that cadmium affects mitochondrial function and ATP production without affecting the $\Delta\Psi_m$ or causing MPT. Thus, ADP-stimulated (state 3) respiration, indicative of the maximum phosphorylation rate, decreased in a dose-dependent manner with increasing cadmium concentrations (ANOVA; $F_{3,21}$=23.3, P<0.0001; Fig. 6). On the other hand, state 4+ respiration, indicative of proton leak, was significantly affected only by the highest cadmium concentration (50 μmol l^{-1}; ANOVA; $F_{3,20}$=12.1, P=0.0001; Fig. 6). RCR of isolated mitochondria decreased with increasing cadmium levels, indicating partial uncoupling of cadmium-exposed mitochondria (ANOVA; $F_{3,21}$=40.4, P<0.0001; Fig. 6). Incubation with 50 μmol l^{-1} Cd^{2+} completely abolished coupling of isolated mitochondria. At higher cadmium levels (100–200 μmol l^{-1}), state 3 and state 4+ respiration was inhibited to less than 5% of the control levels (data not shown), indicating a complete loss of functionality of mitochondria.

Although respiration rate, and especially ADP-stimulated respiration, of oyster mitochondria was strongly affected by cadmium, this metal had no effect on mitochondrial volume or membrane potential. Indeed, no swelling indicative of the MPT was observed in mitochondria exposed to cadmium in the range of 10–1000 μmol l^{-1} (ANOVA; $F_{5,20}$=2.49, P=0.065; Fig. 7A). On the contrary, the absorbance at 520 nm of isolated mitochondria exposed to 10–100 μmol l^{-1} cadmium showed a marginally significant decrease compared with the control mitochondria, indicating that cadmium might induce slight contraction of the mitochondrial volume. Cadmium

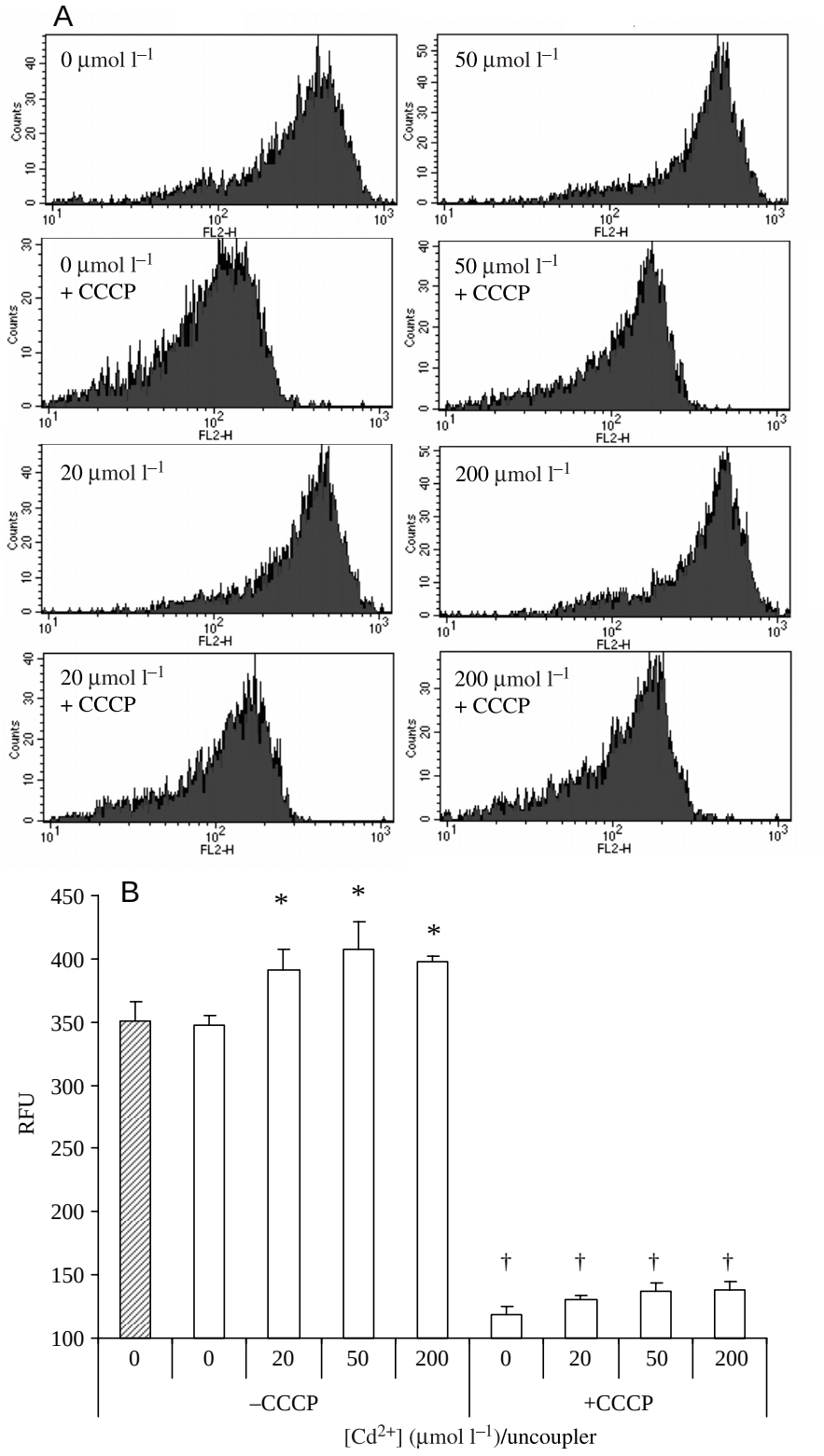

Fig. 3. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) in isolated oyster hemocytes exposed to different cadmium concentrations for 72 h as measured by TMRM staining. (A) Representative distributions of the TMRM fluorescence intensity of control and cadmium-exposed cells before or after treatment with a mitochondrial uncoupler (CCCP). (B) Quantitative graph of the data shown in A indicating the average TMRM fluorescence intensity in control cells and cells exposed to different cadmium concentrations before (–CCCP) and after (+CCCP) exposure to the mitochondrial uncoupler CCCP. The striped bar represents TMRM fluorescence in control hemocytes without digitonin treatment. Vertical bars represent S.E.M. *Values are significantly different from the control (0 μmol l^{-1} Cd^{2+}); †values significantly different from the fluorescence intensity of the respective cell populations in the absence of CCCP (P<0.05; N=6–8).

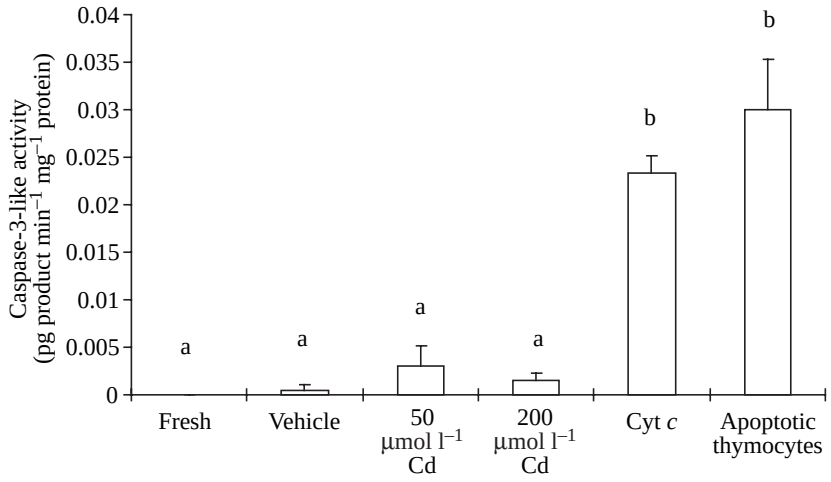

Fig. 4. Caspase-3 activity in freshly isolated oyster hemocytes (Fresh) and hemocytes incubated for 72 h with vehicle, 50 or 200 µmol l^{-1} of cadmium. Cyt *c*: activation of caspase-3 from vehicle-treated hemocytes with cytochrome *c* and dATP. Apoptotic thymocytes: caspase-3 activity in growth-factor-deprived murine thymocytes used as a positive control. Vertical bars represent S.E.M. Values that are not significantly different from each other are denoted by the same letters (P<0.05; N=5–6).

exposure did not affect $\Delta\Psi_m$, as measured by 573/546 fluorescence ratio of TMRM (ANOVA; $F_{4,16}$=2.53, P=0.08; Fig. 7B). *Post-hoc* comparisons showed that the $\Delta\Psi_m$ was slightly below the control only at the highest tested cadmium concentration (50 µmol l^{-1}; P=0.03), which also strongly inhibited respiration of isolated oyster mitochondria (Fig. 6). The mitochondrial uncoupler CCCP, used to collapse $\Delta\Psi_m$, reduced 573/546 fluorescence ratios of mitochondria to similar low levels independent of the cadmium treatment.

Discussion

In vivo, bivalve hemocytes are known to quickly take up and concentrate cadmium from the hemolymph and to retain it internally throughout their circulatory lifespan (McIntosh and Robinson, 1999). This suggests that these cells have limited abilities for cadmium excretion, may concentrate extremely high levels of this metal and may be a key target for cadmium toxicity in oysters. In the present study, we have shown that

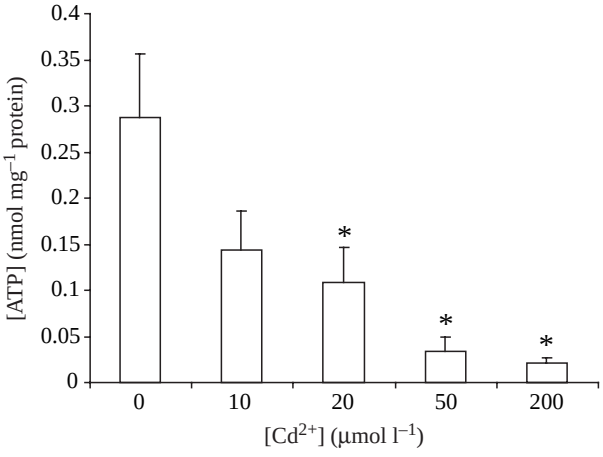

Fig. 5. Changes in the intracellular ATP concentration in oyster hemocytes exposed to different cadmium concentrations for 72 h. Vertical bars represent S.E.M. Asterisks denote values that are significantly different from the control (P<0.05; N=7–13).

cadmium exposure induced apoptosis in oyster hemocytes, as indicated by the typical apoptotic cell morphology and by translocation of phosphatidylserine into the outer leaflet of the cell membrane. The levels of hemocyte apoptosis increased in a dose-dependent manner with increasing cadmium concentration in the range of 0–100 µmol l^{-1} and reached a maximum of 40–45% at 100–200 µmol l^{-1} Cd^{2+} after 72 h of exposure. Higher concentrations of cadmium failed to further increase apoptosis but resulted in elevated necrosis. This switch of the death mechanisms from apoptosis to necrosis with increasing level of cytotoxic insult is typical for the dose–response to cadmium and has been earlier reported in different cell types (Tsangaris and Tzortzatou-Stathopoulou, 1998; De La Fuente et al., 2002; Lopez et al., 2003). After 72 h of exposure to ⩾50 µmol l^{-1} of cadmium, only ~40–50% of oyster hemocytes remained viable. This is likely to be a conservative estimate because it does not take into account the cells that died early during the cadmium exposure and had completely disintegrated and/or were engulfed by the neighboring hemocytes.

Notably, molecular and cellular mechanisms of apoptosis in cadmium-exposed oyster hemocytes differ radically from the cadmium-induced cell death pathways in vertebrate cells, which are the only systems where cadmium-induced apoptosis has been extensively studied. In vertebrates, cadmium exposure results in fast depolarization of the mitochondrial membrane, opening of the mitochondrial permeability pore and the release of cyt *c* into the cytoplasm (reviewed in Wolf and Eastman, 1999). In the cytoplasm, cyt *c* associates with apaf-1 and procaspase-9 to form a complex known as the apoptosome. Formation of this complex activates caspase-9, which is then responsible for activation of caspase-3, the central executioner that facilitates many downstream apoptotic events. Moreover, cadmium addition to isolated mitochondria results in rapid mitochondrial depolarization, the PTP opening and cyt *c* release (Al-Nasser, 2000; Li et al., 2003), suggesting that in vertebrates the MPT may be due to a direct effect of cadmium on mitochondria. This suggestion is further supported by the finding that ruthenium red, which inhibits

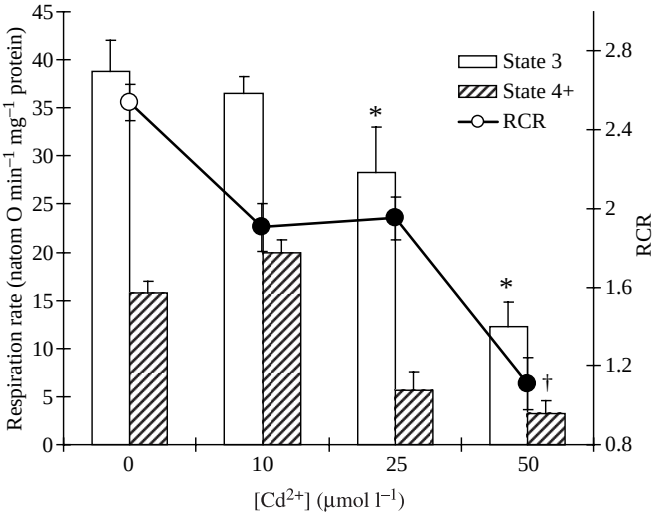

Fig. 6. Effects of cadmium on respiration rate and coupling of isolated oyster mitochondria. State 3, ADP-stimulated respiration; state 4+, respiration in the presence of oligomycin (indicative of proton leak); RCR, respiratory control ratio of state 3 over state 4+ respiration. Vertical bars represent S.E.M. Asterisks, daggers and filled circles denote values of state 3, state 4+ respiration and RCR, respectively, which are significantly different from the control (P<0.05; N=5–6).

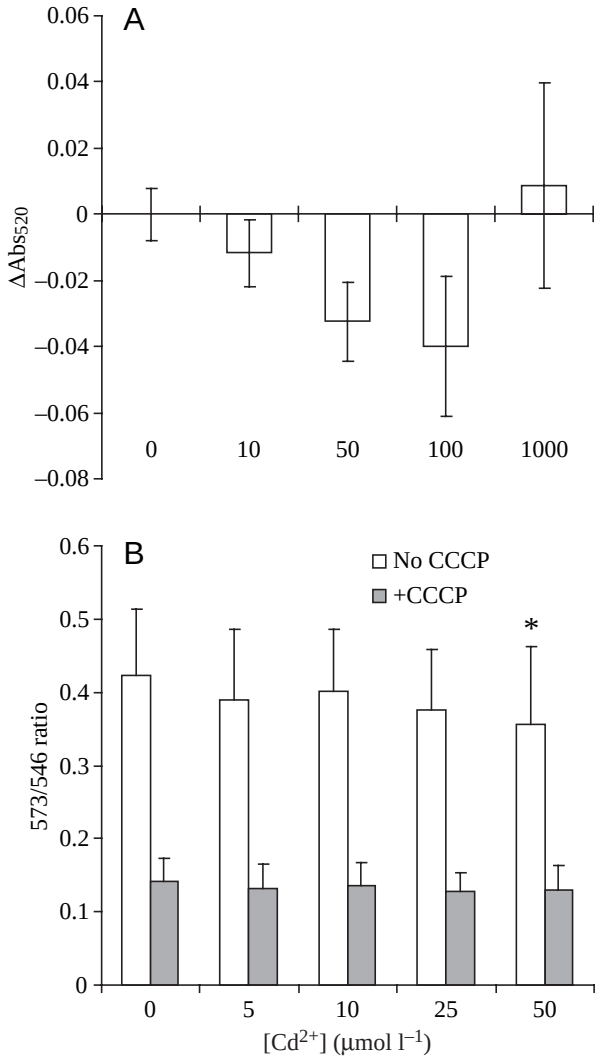

Fig. 7. Effects of cadmium on mitochondrial volume (A) and membrane potential (B) in isolated oyster mitochondria. (A) Mitochondrial swelling was measured as a decrease of absorbance at 520 nm. ΔAbs_{520}, difference in absorbance at 520 nm between control mitochondria and those incubated with different concentrations of cadmium. This method only gives qualitative data about the mitochondrial volume change; positive values of ΔAbs_{520} indicate mitochondrial swelling, while negative values indicate mitochondrial contraction. (B) Mitochondrial membrane potential was measured as a ratio of fluorescence of mitochondria incubated with TMRM at excitation wavelengths of 573 or 546 nm and an emission wavelength of 590 nm. Ratio of fluorescence at 573 nm over 546 nm excitation wavelengths is linearly proportional to membrane potential ($\Delta\Psi_m$). Vertical bars represent S.E.M. Asterisks denote values that are significantly different from the control (P<0.05; N=5–6).

cadmium transport into mitochondria, also prevents PTP opening and the loss of transmembrane potential (Li et al., 2003). Mitochondrial depolarization in response to cadmium, with the concomitant release of cyt *c* and activation of caspase-3-like enzymes, has been demonstrated in all mammalian cell types studied to date, including kidney cells, hepatocytes, neurons and, most relevant, immune cells, suggesting that this is a universal mechanism of cadmium-induced apoptosis in mammals (Habeebu et al., 1998; Kim et al., 2000; Galan et al., 2001; Kondoh et al., 2001, 2002; Wätjen et al., 2002; Aydin et al., 2003; Shih et al., 2004). By contrast, cadmium-induced apoptosis in oyster hemocytes is activated by a pathway fundamentally different from that of the vertebrates and independent of MPT. In the present study, there was clearly no loss of the $\Delta\Psi_m$ in response to cadmium exposure either in intact oyster hemocytes or in isolated oyster mitochondria, strongly suggesting there was no opening of the PTP. Moreover, mitochondrial swelling, which is another indicator of the MPT (Li et al., 2003), was not induced in oyster mitochondria by even very high levels of cadmium (100–1000 μmol l^{-1}), even though they completely abolished mitochondrial respiration and coupling. In fact, the $\Delta\Psi_m$ was slightly elevated in cadmium-treated oyster hemocytes, indicating hyperpolarization of mitochondrial membrane. This hyperpolarization could be due to the inhibition of mitochondrial ATP synthesis in cadmium-treated hemocytes, as indicated by the strong dose-dependent inhibition of the ADP-stimulated respiration in cadmium-treated mitochondria and by the decreased ATP levels in cadmium-exposed oyster cells. The mechanisms of this cadmium-induced inhibition of ATP synthesis are unknown and may be due to the inhibition of the F_0/F_1-ATPase and/or reduced ADP transport into the mitochondria. Indeed, the respiration rate of 'resting' mitochondria in the presence of oligomycin was not significantly affected by cadmium except at the highest test concentration (50 μmol l^{-1}), indicating that the electron transfer chain was not inhibited by the low cadmium levels that inhibited ADP-dependent respiration. Proton conductance

through the F_0/F_1-ATPase during ATP production would dissipate the membrane potential, and, therefore, inhibition of ATP synthesis can conceivably result in hyperpolarization of the mitochondrial membrane (Brand, 1995).

No significant activation of caspase-3-like activity was detected in cadmium-treated apoptotic hemocytes, supporting our conclusion that cadmium-induced apoptosis in oysters does not involve the MPT and formation of the apoptosome. However, caspase-3-like activity could be activated in hemocyte extracts by incubating with dATP and cyt *c*. Levels of the cyt *c*-activated caspase-3 activity in oysters were comparable with caspase-3 activity in apoptotic rat thymocytes, indicating that the enzyme was present in oyster cells in significant quantities. The lack of activation of caspase-3 *in vivo* in cadmium-exposed oyster hemocytes suggests that cyt *c* was not released from mitochondria during cadmium exposure.

Another notable difference between cadmium-induced apoptosis in oysters and mammalian models is the change in energy status, particularly ATP levels, during apoptosis. Apoptosis is an energy-requiring process, and earlier studies on mammalian models have demonstrated that depletion of cellular ATP levels resulted in a switch of the mode of death from apoptosis to necrosis (Lopez et al., 2003; Nieminen, 2003). In oyster hemocytes, we found a decrease in the cellular ATP levels with increasing cadmium levels. Importantly, a significant drop in ATP levels was detected in the apoptotic range of cadmium concentrations (20–50 μmol l^{-1}), indicating that low ATP levels *per se* do not shift the form of cell death towards necrosis in these cells. A characteristic feature of the cellular physiology of intertidal mollusks, including oysters, is their capability for metabolic rate depression under conditions of environmental stress (Hochachka and Guppy, 1987; Hand and Hardewig, 1996; Storey, 1988). It has been shown that a variety of stressors, particularly those that affect ATP production such as hypoxia/anoxia, subfreezing and freezing temperatures, severe osmotic stress, result in a drastic downregulation of the use of ATP to 5–20% of the normal levels in marine mollusks (Storey and Storey, 1990; Storey and Churchill, 1995; Sokolova et al., 2000; Sokolova and Pörtner, 2001). This stress-induced decrease in ATP turnover and cellular energy demand may be important in allowing some crucial energy-dependent processes (e.g. apoptosis) to proceed despite a decline in the overall cellular ATP levels. However, a further decrease in ATP levels will eventually result in the situation when the free Gibbs energy of ATP hydrolysis is insufficient to support functioning of cellular ATPases (Sokolova et al., 2000), thus resulting in necrotic cell death. Although no data are currently available about the effects of cadmium exposure on the ATP turnover rates in oysters, extrapolation from a wide variety of other stressors that affect mitochondrial ATP production suggests that metabolic rate depression is also likely to occur in cadmium-exposed oyster cells. Further studies are required to elucidate the relationship between metabolic rate regulation and the energy partitioning for apoptotic processes under conditions of cadmium exposure in oysters.

The absence of mitochondrial involvement in apoptosis in response to cadmium is a unique feature of oyster cells that sets them apart from other systems where cadmium-induced apoptosis has been studied. However, alternative pathways of apoptosis, which do not involve MPT, are not unknown and can be induced by some stimuli in other organisms. In vertebrates, Mn^{2+} was shown to induce apoptosis without MPT (Oubrahim et al., 2001), whereas other metals such as Cd^{2+}, Cu^{2+}, Zn^{2+} and arsenic result in the MPT (Robertson and Orrenius, 2000; De La Fuente et al., 2002; Wätjen et al., 2002; Pulido and Parrish, 2003). In *Drosophila* and *Caenorhabditis elegans*, mitochondria did not release cyt *c* or undergo a decrease in $\Delta\Psi_m$ during apoptosis induced by a variety of stimuli including UV radiation and oxidative stress (Varkey et al., 1999; Zimmermann et al., 2002; Claveria and Torres, 2003), although other apoptotic changes, such as translocation of phosphatidylserine to the outer surface of the cell membrane, were conserved (Zimmermann et al., 2002). In contrast to oysters and vertebrates, cyt *c* added directly to the *Drosophila* or *C. elegans* cell extracts failed to significantly activate caspase-3 (Kanuka et al., 1999). Our data suggest that, unlike insects and nematodes, oysters possess the cellular potential for caspase-3 activation and for the amplification of the apoptotic signal through the mitochondria-dependent pathways, but these pathways are not activated during cadmium-induced apoptosis. Further investigation will elucidate what types of apoptotic stimuli can induce a drop in the $\Delta\Psi_m$ and activate caspase activity in oysters.

Regardless of the mechanism, it seems likely that elevated hemocyte mortality due to cadmium-induced apoptosis and necrosis could significantly impair the cellular defense ability of oysters in much the same way that diseases which result in the elimination of human immune cells (such as AIDS) result in immunodeficiency. Field studies of hemocyte numbers in heavy-metal-exposed oyster populations have yielded controversial results. Some researchers have reported decreased numbers of circulating hemocytes in oysters exposed to pollutants in the field or the lab, whereas others have shown elevated levels or no differences from control oyster populations (Suresh and Mohandas, 1990; Pipe et al., 1999; Dyrynda et al., 2000; Oliver et al., 2001). Interestingly, changes in the DNA profile of hemocytes similar to those seen during apoptosis have been reported for several bivalve populations from contaminated sites (Bihari et al., 2003), suggesting elevated levels of DNA damage and possibly apoptosis *in situ*. It is possible that an increase in cell death is compensated for by the increased proliferation and differentiation of immune cells. Compensation for elevated cell mortality in oysters exposed to high cadmium levels, if it occurs *in vivo*, would result in elevated hemocyte turnover and the associated energetic costs. There are no studies to date on the turnover rates of oyster hemocytes from heavy-metal-polluted and unpolluted areas.

It is worth noting that the number of circulating hemocytes does not necessarily reflect the total size of the hemocyte population and can change over a short time span due to the

dynamic association/dissociation between hemocytes and oyster tissues (Ford et al., 1993). Therefore, the number of circulating hemocytes in heavy-metal-exposed oysters may not be a reliable estimate of hemocyte mortality. Our study suggests that the level of apoptosis in hemocytes may be a better indicator of the general viability and health of the hemocyte population in oysters compared with the simple enumeration of hemocytes in circulation and thus may be useful as a biomarker of immunotoxic and cytotoxic effects of heavy metals in bivalves. Currently, field studies are underway to test the applicability of apoptosis as a biomarker of immunotoxic effects of heavy metal pollution in oyster populations.

In summary, cadmium exposure in oysters results in significant cytotoxicity and considerably elevated levels of apoptosis and necrosis in oyster hemocytes. High rates of apoptosis due to cadmium exposure may have important implications for the immune defense of the oysters, resulting in the weakened immune system due to hemocyte loss. Mitochondria are likely to be a key intracellular target for cadmium cytotoxicity in oysters, as indicated by the inhibition of the phosphorylation rate, decreased mitochondrial coupling and depletion of the intracellular ATP levels. However, unlike vertebrate mitochondria, mitochondria of oysters do not undergo permeability transition in response to cadmium. This emphasizes variability of evolutionary pathways of the programmed cell death in response to the same stimulus in different organisms and cautions against extrapolation of cellular biomarkers of heavy metal toxicity developed for vertebrates to invertebrate species.

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