Cadmium affects metabolic responses to prolonged anoxia and reoxygenation in eastern oysters (*Crassostrea virginica*)

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Submitted 9 June 2009; accepted in final form 31 August 2009

Kurochkin IO, Ivanina AV, Eilers S, Downs CA, May LA, Sokolova IM. Cadmium affects metabolic responses to prolonged anoxia and reoxygenation in eastern oysters (*Crassostrea virginica*). *Am J Physiol Regul Integr Comp Physiol* 297: R1262–R1272, 2009. First published September 2, 2009; doi:10.1152/ajpregu.00324.2009.—Benthic marine organisms such as mollusks are often exposed to periodic oxygen deficiency (due to the tidal exposure and/or seasonal expansion of the oxygen-deficient dead zones) and pollution by metals [e.g., cadmium, (Cd)]. These stressors can strongly affect mollusks’ survival; however, physiological mechanisms of their combined effects are not fully understood. We studied the effects of Cd exposure on mitochondrial metabolism, abolishes reoxygenation-induced stimulation of mitochondrial respiration, and ADP-stimulated (state 3) oxygen uptake by mitochondria (MO2), but no change in the resting (state 4) MO2 of oyster mitochondria, along with a slight but significant reduction of mitochondrial respiratory control ratio. During reoxygenation, there was a significant overshoot of mitochondrial MO2 (by up to 70% above the normoxic ratory control ratio). During reoxygenation, there was a significant overshoot of mitochondrial MO2 (by up to 70% above the normoxic ratory control ratio). However, physiological mechanisms of their combined effects are not fully understood. We studied the effects of Cd exposure on mitochondrial function. Oysters can survive frequent bouts of anoxia-tolerant intertidal mollusks *Crassostrea virginica* (eastern oysters). Anoxia led to an onset of anaerobiosis indicated by accumulation of l-alanine, acetate, and succinate. Prolonged anoxia (for 6 days) caused a decline in the maximum activity of electron transport chain and ADP-stimulated (state 3) oxygen uptake by mitochondria (MO2), but no change in the resting (state 4) MO2 of oyster mitochondria, along with a slight but significant reduction of mitochondrial respiratory control ratio. During reoxygenation, there was a significant overshoot of mitochondrial MO2 (by up to 70% above the normoxic steady-state values) in control oysters. Mild mitochondrial uncoupling during prolonged shutdown in anoxic tissues and a subsequent strong stimulation of mitochondrial flux during recovery may help to rapidly restore redox status and protect against elevated reactive oxygen species formation in oysters. Exposure to Cd inhibits anaerobic metabolism, abolishes reoxygenation-induced stimulation of mitochondrial MO2, and leads to oxidative stress (indicated by accumulation of DNA lesions) and a loss of mitochondrial capacity during postanoxic recovery. This may result in increased sensitivity to intermittent hypoxia and anoxia in Cd-exposed mollusks and will have implications for their survival in polluted estuaries and coastal zones.

Periodical oxygen deficiency is an important environmental stressor in intertidal and coastal habitats. Short-term intermittent hypoxia/anoxia (from several hours to several days, depending on the state of the tides) often occurs in intertidal invertebrates during the low tide, and may also occur in tidal pools and shallow lagoons with limited water exchange (8, 36, 49). Furthermore, long-term severe hypoxia and anoxia triggered by anthropogenic release of nutrients has become a serious issue in many estuaries and coastal zones rivaling the climate change (18, 22). In the coastal dead zones, benthic invertebrates including mollusks can be exposed to severe hypoxia or anoxia for prolonged periods (from weeks to up to several months) (10, 22, 34, 46). Long-term oxygen deficiency often leads to the massive die-offs in species with limited or no motility, such as mollusks, but even short hypoxic and anoxic bouts (for only several days) can have long-term fitness costs, such as reduced growth and reproduction (18, 19, 56).

Intertidal mollusks including oysters are among the animal champions of hypoxia and anoxia tolerance. They possess a suite of physiological and biochemical adaptations to periodic oxygen deprivation that involves shutdown of aerobic metabolism and transition to anaerobiosis, along with a strong reduction in overall metabolic rate known as metabolic rate depression (31, 70). Metabolic pathways of anaerobic ATP production and cellular mechanism of metabolic rate depression are well characterized in hypoxia-tolerant invertebrates including mollusks (27, 30, 31, 54, 67, 69, 70). In contrast, alterations of mitochondrial functions during intermittent anoxia and potential adaptations that allow mollusks to rapidly resume aerobic function upon reoxygenation have not been extensively studied and are not well understood. This aspect of metabolic adaptations to anoxia is especially intriguing given that transitions between oxygen-deprived and normoxic conditions can result in mitochondrial dysfunction and elevated production of reactive oxygen species (ROS) leading to extensive tissue-wide damage (6, 11, 41, 58); yet many marine intertidal mollusks are able to survive such transitions without any apparent detrimental effects.

Periodical oxygen deprivation in intertidal and coastal habitats often co-occurs with other stressors, such as anthropogenic pollution, that may strongly affect energy metabolism of marine mollusks. Cadmium (Cd) is a common pollutant that can accumulate in coastal and estuarine environments from both natural and anthropogenic sources (23, 24, 57). One of the key aspects of Cd toxicity is its strong inhibitory effect on aerobic metabolism and mitochondrial function that results in cellular energy deficiency and oxidative stress, and at high Cd levels, leads to the functional collapse of mitochondria and apoptosis (9, 12, 38, 39, 61, 68, 71). As a result, profound effects of Cd on organisms’ metabolic performance are expected. However, it is not known whether Cd-induced impairment of mitochondria may affect mitochondrial responses to oxygen deficiency and their recovery during reoxygenation in intertidal mollusks.

Eastern oysters (*Crassostrea virginica*) are an excellent model to study the effects of intermittent anoxia and Cd stress on mitochondrial function. Oysters can survive frequent bouts...
of oxygen deficiency during low tides, as well as prolonged (days to weeks) periods of oxygen deprivation during neap tides in the intertidal zone or during seasonal hypoxia in the coastal dead zones (8, 49). Like many marine bivalves, oysters can concentrate Cd in soft tissues making these mollusks susceptible to Cd toxicity (23, 53, 57). Our earlier studies showed that oyster mitochondria accumulate high levels of Cd and are highly susceptible to toxic and pro-oxidant effects of this metal (12, 61, 63, 66). Therefore, both intermittent anoxia and Cd exposure are environmentally relevant stressors for oysters and can directly affect their energy metabolism.

The goal of this study was to determine the effects of intermittent anoxia (achieved by air exposure) and subsequent normoxic recovery on aerobic and anaerobic metabolism and oxidative stress and to test whether Cd stress modifies metabolic responses to intermittent oxygen deprivation in eastern oysters (C. virginica). For this, we determined the effects of prolonged anoxia and subsequent recovery on mitochondrial function and capacity (including oxidation rates of different substrates, phosphorylation efficiency, and respiratory control), accumulation of anaerobic end products and oxidative DNA damage in control and Cd-exposed oysters. Given the ability of molluscan mitochondria to oxidize a broad range of metabolic intermediates (2–4) and the fact that anoxia exposure may result in a significant change of intracellular abundance of these intermediates (16, 27, 43), we also tested whether mitochondrial substrate preferences for three major classes of mitochondrial substrates (amino acids, fatty acids, and short-chain organic acids) shift during anoxia and reoxygenation, reflecting changes in the relative abundance of these substrates.

MATERIALS AND METHODS

Animal collection and maintenance. Adult oysters (C. virginica; 7- to 12-cm shell length) were obtained from Taylor Shellfish Farms (Shelton, WA). Oysters were shipped within 24 h to the University of North Carolina at Charlotte and placed in recirculated aerated tanks with artificial seawater (ASW) (Instant Ocean, Kent Marine, Acworth) at 20 ± 1°C and 30 ± 1‰ salinity for 2 wk. Our previous studies have shown that this temperature and salinity are close to the optima for oysters (Sokolova IM, unpublished data). After the preliminary acclimation, experimental oysters were randomly divided into six groups, and each group was placed in a separate water tank. Three groups continued in Cd-free ASW, and three were exposed 50 μg/l Cd (as CdCl₂) in ASW for 30 days. We used a static-renewal regimen described in our earlier studies that allowed maintaining Cd concentrations close to the target levels of 50 μg/l Cd (39). This Cd concentration is within the range found in polluted estuaries (15–80 μg/ml) (23). Our previous studies also showed that 30–60 days of exposure to 50 μg/l Cd result in physiologically relevant tissue burdens of Cd similar to those found in oysters from polluted estuaries (13, 66 and references therein). Thus, Cd exposure conditions used in this experiment represent environmentally relevant levels for oysters.

To mimic environmental anoxia in the intertidal zone, control and Cd-exposed oysters were exposed to air. Oysters were randomly selected from each of the three replicate tanks set up for control or Cd-exposed oysters, placed into plastic trays lined with seawater-soaked paper towels, and their shells were closed with a rubber band to prevent gaping in the air. Subsamples of oysters were collected after 1, 3, and 6 days of air exposure for mitochondrial isolation and tissue metabolite determination. It is worth noting that, while typical periods of air exposure for oysters in the intertidal zone are several hours, they may periodically experience more prolonged air exposures (lasting for several days) during neap tides (8, 49). Therefore, while a 6-day exposure to environmental anoxia represents an extreme stress for oysters, it is within the environmentally relevant range for this species. After 6 days of anoxia, a subsample of oysters was returned into tanks with well-aerated ASW and allowed to recover for 12–24 h. Recovery was conducted at the same Cd levels as during the acclimation period (0 and 50 μg/l Cd for control and Cd-exposed oysters, respectively). Samples for mitochondrial isolation and tissue metabolite determination were taken after 1, 6, and 12 h of recovery, while hemolymph oxygen levels were continuously monitored during 24 h of postanoxic recovery. All exposures were carried out at 20 ± 2°C and repeated 3–4 times with separate subsets of experimental animals to obtain a sufficient amount of tissues for all analyses. Oysters were fed three times a week ad libitum (except during the air exposure) with a commercial algal blend containing Nanochloropsis, Phaeodactylum tricornutum, and Chlorella (DT’s Live Marine Plankton, DT’s Plankton Farm, Sycamore, IL).

Hemolymph oxygen partial pressure (PO₂) was monitored online by implanted needle-type oxygen microsensors with integrated temperature compensation (Tx-Type, PreSens, Regensburg, Germany). Oxygen sensors were calibrated in oxygen-free [0%] using sodium sulfite (Na₂SO₃) and air-saturated (100%) seawater and was inserted into pericardium through a small hole in the right shell valve as described elsewhere (38). For optimum positioning of the microsensor inside the pericardial cavity, the syringe was placed on the right shell valve and fixed with dental periphery wax and SuperGlue to avoid disruption of mantle and pericardium by the sensor movement. Values of hemolymph PO₂ were recorded in normoxia, during air exposure and 24 h of reoxygenation as %air saturation and converted to PO₂ as follows: hemolymph PO₂ (kPa) = (P_{\text{air}} - P_{\text{H2O}}) × 0.2095 × (% air saturation/100), where P_{\text{air}} is atmospheric pressure (kPa), P_{\text{H2O}} is temperature-specific water vapor pressure (kPa) calculated after Dejours (17), and 0.2095 is the proportion of oxygen content in the air.

Respiratory time activity (RTA) was determined as percentage of time an oyster spent in fully open (and presumably ventilating) state using visual surveillance and hemolymph PO₂ recordings as described elsewhere (39). Typically, spontaneous shell closure (corroborated by visual surveillance) caused a dramatic reduction of hemolymph PO₂, indicating cessation of ventilation. Therefore, during normoxia and postanoxic recovery only PO₂ values recorded during the periods when oysters were open were taken into consideration. During air exposure, shell closure resulted in a decrease of hemolymph PO₂ to zero, indicating anoxia (Fig. 1).

Mitochondrial oxygen consumption. Mitochondria were isolated from oyster gills using a method modified from Sokolova (61). Briefly, 6–8 g of oyster gills were homogenized in the ice-cold buffer containing 100 mM sucrose, 200 mM KCl, 100 mM NaCl, 8 mM EGTA, and 30 mM HEPES, pH 7.5. Mitochondria were obtained by differential centrifugation; the homogenate was centrifuged at 4°C for 8 min to remove cell debris, and the supernatant was centrifuged at 8,500 g for 8 min to obtain a mitochondrial pellet. The mitochondrial pellet was surface washed with homogenization buffer and resuspended in 2 ml of ice-cold assay medium consisting of 150 mM sucrose, 250 mM KCl, 10 mM glucose, 10 mM KH₂PO₄, 1 mM MgCl₂, 10 mg/ml BSA (fatty acid free), and 30 mM HEPES, pH 7.2.

Oxygen uptake by mitochondria (MO₂) was measured in 1 ml water-jacketed, temperature-controlled chambers using Clarke-type oxygen electrodes (Qubit Systems, Kingston ON, Canada) at 20°C as described in Sokolova (61). To test the effects of anoxia and reoxygenation on mitochondrial capacity, mitochondrial MO₂ was measured in the presence of 3.2 mM pyruvate (with 0.5 mM malate to spark oxidation), 10 mM succinate, or 0.5 mM N.N.N.’-tetramethyl-p-phenylene diamine (TMPD) in the presence of 1 mM ascorbate. Pyruvate is an NADH-coupled substrate donating electrons at mitochondrial complex I, whereas succinate and TMPD directly donate electrons to mitochondrial complexes II and III, respectively. Respiration with succinate and TMPD was measured in the presence of 2.5 μg/ml rotenone and 1 μM antimycin, respectively, to inhibit.
perchloric acid with 150 mM EDTA for the maximum recovery of nitrogen and homogenized with four volumes of ice-cold 0.6 M nitrogem. Tissue was powdered with mortar and pestle under the liquid mitochondrial protein as described in Sokolova (61).

the upstream complexes of the electron transport chain (ETC). The maximum oxidation activity of ETC was measured in the presence of an uncoupler, 100 μM 2,4-dinitrophenol (DNP) with succinate and pyruvate as substrates. For determination of mitochondrial substrate preferences, we isolated mitochondria from control (non-Cd-exposed) oysters: 1) maintained in normoxia, 2) after 6 days of anoxic exposure, and 3) after 1 h recovery following 6 days in anoxia. Mitochondrial respiration was measured using saturating concentrations of fatty acid-linked substrates (10 μM palmitoyl-l-carnitine or myristoyl-l-carnitine), glycolysis-linked substrates (10 mM succinate, 1 mM malate, or 3.2 mM pyruvate) and amino acids (10 mM L-glutamate or L-alanine). Malate (0.5 or 0.2 mM) was used to spark oxidation of pyruvate or amino acids, respectively; no sparker was required for fatty acid oxidation by oyster mitochondria. Mitochondrial MO2 was measured at saturating O2 concentrations according to the standard practice in mitochondrial physiology. All assays were completed within 2 h of isolation of the mitochondria.

State 3 (ADP-stimulated), state 4 (resting), and state 4ol (in the presence of 2.5 μg/ml oligomycin) respiration were determined as described earlier (61). State 3 of mitochondria respiration was achieved by the addition of 100–150 μM of ADP. States 4 and 4ol MO2 represent uncoupled respiration when most (or all, in the case of state 4ol) energy of substrate oxidation is used to counteract the futile proton and cation cycles (collectively known as proton leak) across the mitochondrial membrane (7, 60). The ADP/O ratio was determined as a measure of phosphorylation efficiency of mitochondria utilizing different substrates (21). Respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 respiration and was used as an index of mitochondrial integrity (21, 60). Protein concentrations in mitochondrial suspensions were measured using a modified Biuret method with 0.1% Triton X-100 (5). Average concentration of mitochondrial protein in the respiratory chambers was 3–5 mg/ml. Mitochondrial respiration rates were corrected for the electrode drift and nonmitochondrial respiration and converted into nmath O\textsuperscript{-1} mg\textsuperscript{-1} mitochondrial protein as described in Sokolova (61).

Tissue metabolite concentrations. Gills and adductor muscles of oysters were quickly excised and immediately shock frozen in liquid nitrogen. Tissue was powdered with mortar and pestle under the liquid nitrogen and homogenized with four volumes of ice-cold 0.6 M perchloric acid with 150 mM EDTA for the maximum recovery of tissue ATP (62). Precipitated protein was removed by centrifugation. The extract was neutralized with 5 mM potassium hydroxide to pH 7.2–7.5. Precipitated potassium perchloride was removed by a second centrifugation. Extracts were stored at –80°C.

Concentrations of all metabolites were measured in neutralized perchloric acid extracts spectrophotometrically by using enzymatic tests described elsewhere (5, 26). For determination of phospho-l-arginine (PLA) levels, PLA in the sample was subjected to acid hydrolysis yielding 1-arginine, and PLA levels were calculated as a difference in l-arginine content of the sample before and after PLA hydrolysis (51). Succinate content was determined using a succinic acid test kit (R-Biopharm, Darmstadt, Germany). Enzymes, substrates, and cofactors for determination of all other metabolites were purchased from Roche (Indianapolis, IN) or Sigma Aldrich (St. Louis, MO).

Oxidative DNA damage. Gill tissues from control and Cd-exposed oysters from normoxic conditions, 6 days of hypoxia, and 1 h reoxygenation were ground to a fine powder consistency in liquid nitrogen. Approximately 50 mg of frozen, powdered sample was placed in a microcentrifuge tube, and DNA was isolated using a Getyl DNA-Cell, tissue kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. DNA concentration was determined with an Inviron/Molecular Probes Quant-iT DNA Assay Kit, Broad Range (Invitrogen, Carlsbad, CA) using a Qubit fluorometer (Invitrogen, Carlsbad, CA). Concentration of apurinic/apyrimidinic (AP) sites in DNA was determined using the Dojindo DNA Damage Quantification Kit-AP Site Counting (Dojindo Laboratories) and was conducted according to the manufacturer’s instructions with a slight modification; instead of colorimetric reporting, we used NEN Western lightning chemiluminescence (Perkin Elmer, Waltham, MA), which increases the sensitivity of the assay by more than 10-fold. Chemiluminescence signal was measured using a Bio-Tek FL800 fluorescent microplate reader (Bio-Tek, Winooski, VT).

Chemicals. All other chemicals were purchased from Sigma Aldrich, Roche, or Fisher Scientific (Pittsburg, PA) and were of analytical grade or higher.

Statistics. Statistical analysis was performed using ANOVA after testing for normality of the data distribution and homogeneity of variances, and was followed by post hoc procedures (Fisher’s least significant difference test for unequal N). For hemolymph PO2 and RTA, repeated-measures ANOVA was used, with individual oysters as a repeated-measure variable. For all other end points, generalized linear model ANOVA was used. Factor effects and differences between the means were considered significant if the probability of Type I error was < 0.05. Statistical analyses were always conducted on the raw values of the studied endpoints; however, for easier graphical comparison, the values were converted to percentage of control values for mitochondrial respiration and hemolymph PO2. Data are presented as means ± SE.

RESULTS

Hemolymph oxygenation. Under normoxic conditions, hemolymph oxygenation (PO2) was significantly higher in Cd-exposed oysters compared with their control counterparts (17.9 ± 0.7 kPa vs. 10.2 ± 2.3 kPa, respectively, P < 0.05).

Hemolymph oxygenation dropped to nearly 0 kPa within 10–30 min after shell closure, and hemolymph remained essentially anoxic throughout 6 days of air exposure. Dynamics of the hemolymph reoxygenation significantly differed in control and Cd-exposed oysters (P < 0.001 for interaction of the factors Cd and anoxia/recovery exposure). In control oysters, hemolymph PO2 increased to 135% of preanoxic levels during the first 12 h of recovery and reached 154% of preanoxic levels during prolonged (13–24 h) recovery (Fig. 1). In contrast, hemolymph oxygenation of Cd-exposed oysters remained sig-
nificantly reduced (at 66–48% of preanoxic levels) throughout the recovery period (Fig. 1). RTA significantly increased during postanoxic recovery. During normoxia, oysters spent on average 55–78% of their time open and ventilating, while during postanoxic recovery this percentage increased to 92–96% within the first 5 h of recovery and remained elevated (at 83–87%) up to 12 h of recovery (longer times not tested) \((P < 0.01)\). There was no difference in RTA between control and Cd-exposed oysters in normoxia or during recovery \((P > 0.05)\).

Mitochondrial capacity. Mitochondrial \(\text{MO}_2\), efficiency (as indicated by \(\text{ADP}/\text{O}\) ratios) and respiratory control ratios significantly differed depending on the substrate being oxidized. \textit{State 3} (ADP-stimulated) respiration was fastest in mitochondria respiring on glutamate and lowest with alanine and malate (Fig. 2A), whereas \textit{state 4} (resting) respiration was fastest with succinate compared with all other substrates (Fig. 2B). Mitochondrial phosphorylation efficiency (indicated by \(\text{ADP}/\text{O}\) ratios) increased in order: succinate \(<\) glutamate \([\text{pyruvate} = \text{myristoyl carnitine} = \text{malate}] < [\text{alanine} = \text{palmitoyl carnitine}]\) (Fig. 2D). RCR was also the lowest in mitochondria respiring on succinate and the highest with glutamate (Fig. 2C). There was no marked preference in terms of oxidation rate or mitochondrial efficiency toward a certain group of substrates (glycolysis-linked ones, amino acids or fatty acids). Exposure to anoxia (for 6 days) and/or reoxygenation (for 1 h) did not significantly affect preferential substrate oxidation as indicated by the absence of significant interactions between the effects of substrate and anoxia/reoxygenation exposure on \textit{states 3} and \textit{4} and RCR \((P > 0.05)\). However, \(\text{ADP}/\text{O}\) ratios of different substrates were differentially affected by anoxia/reoxygenation \((P < 0.05\) for interactive effects of substrate and anoxia/reoxygenation exposure). Specifically, there was a slight but significant change in \(\text{ADP}/\text{O}\) ratio for pyruvate that decreased from 2.05 in control to 1.71 after 6 days anoxia, returning to the control levels (2.03) within 1 h of reoxygenation \((P < 0.05)\). For all other substrates, \(\text{ADP}/\text{O}\) ratios did not change in response to anoxia or reoxygenation.

Oxygen consumption rates (\(\text{MO}_2\)) were significantly higher in Cd-exposed oysters compared with the controls with all three tested substrates (pyruvate, succinate and TMPD) (Fig. 3). However, RCR was significantly lower in Cd-exposed oysters suggesting elevated rates of proton leak and partial uncoupling (RCR

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**Fig. 2.** Substrate-dependent oxygen consumption of isolated mitochondria from \textit{Crassostrea virginica}. Data shown for mitochondria respiring on different substrates. ADP-stimulated \textit{(state 3)} and resting \textit{(state 4)} respiration rates are shown for mitochondria isolated from control and Cd-exposed oysters maintained under normoxic conditions. \textit{A}: \textit{state 3} respiration, \textit{B}: \textit{state 4} respiration, \textit{C}: \textit{R}espiratory control ratio (RCR), \textit{D}: \textit{ADP}/\text{O} ratio. Columns that do not share a letter represent values significantly different from each other \((P < 0.05); n = 3–7\).

**Fig. 3.** Mitochondrial oxygen consumption of isolated mitochondria from control and Cd-exposed \textit{C. virginica} respiring on different substrates. ADP-stimulated \textit{(state 3)} and resting \textit{(state 4)} respiration rates are shown for mitochondria isolated from control and Cd-exposed oysters maintained in normoxia. \*Values significantly different between mitochondria from control and Cd-exposed oysters \((P < 0.05); n = 6–8\).
with pyruvate: 4.5 ± 0.2 vs. 3.8 ± 0.2, and succinate: 3.0 ± 0.1 vs. 2.6 ± 0.2 in control and Cd-exposed oysters, respectively; *P < 0.05.

Anoxic exposure resulted in a notable (albeit not statistically significant) decrease in state 3 respiration in mitochondria of control and Cd-exposed oysters, whereas state 4 respiration rate did not change (Fig. 4). In contrast, mitochondrial response to postanoxic reoxygenation differed between control and Cd-exposed oysters. In control oysters, mitochondrial respiration was significantly elevated during the first 6 h of reoxygenation, gradually returning to the control levels, while in Cd-exposed oysters there was no overshoot of mitochondrial respiration during postanoxic recovery (Fig. 4). State 3 respiration of mitochondria from Cd-exposed oysters remained depressed below the normoxic steady-state levels throughout the recovery period, whereas state 4 respiration rates were not affected (Fig. 4). The above-described patterns of mitochondrial responses to hypoxia and reoxygenation were the same regardless of the substrate oxidized by mitochondria (pyruvate, succinate or TMPD+ascorbate). We have also measured state 4ol respiration (in the presence of oligomycin) in mitochondria of control and Cd-exposed oysters with succinate and pyruvate as the substrates. State 4ol respiration was on average 25% lower than the respective state 4 and changed in the same way as state 4 respiration (data not shown). In control oysters, RCR significantly decreased during prolonged anoxia and recovery (Fig. 4). Mitochondria of Cd-exposed oysters had lower RCR under normoxic and anoxic conditions than the controls and experienced a further reduction in RCR during postanoxic recovery (Fig. 4). In both control and Cd-exposed oysters, the

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**Fig. 4.** Effect of intermittent anoxia (during air exposure) and recovery on respiration and RCR of isolated mitochondria from *C. virginica.* Data are shown for mitochondria respiring with pyruvate (A, C, and E) and succinate (B, D, and F). A and B: state 3 (ADP-stimulated) respiration; C and D: state 4 (resting) respiration; E and F: RCRs. The anoxia- and reoxygenation-induced patterns of change in state 3 and state 4 respiration with tetramethylpentadecane (TMPD) and ascorbate as electron donors were similar to those with pyruvate and succinate and are not shown. State 4ol respiration with succinate and pyruvate in the presence of oligomycin was on average 25% lower than the respective state 4 and changed in the same way as state 4 respiration (data not shown). †Values that are significantly different between control (non-Cd-exposed) and Cd-exposed oysters. MO2, oxygen uptake by mitochondria. *Significantly different from the respective normoxic values (*P < 0.05). aValues differed between postanoxic recovery and 6 days of anoxia (P < 0.05). For state 3 and 4 respiration, values are expressed as %respective normoxic controls (see Fig. 3 for the absolute values); n = 6–8.
lowest RCR values were attained after 12 h of postanoxic recovery.

To test whether the observed increase in states 3 and 4 MO$_2$ during postanoxic recovery of control oysters was due to the changes in the maximum oxidation capacity of mitochondrial ETC, we measured respiration rates in DNP-uncoupled mitochondria. The maximum oxidation rate of ETC was significantly reduced during 6 days of anoxia and returned to the control levels after 1 h of recovery (Fig. 5). No overshoot in DNP-uncoupled respiration was detected during postanoxic recovery.

**Anaerobic metabolism.** Anaerobic end products accumulated during anaerobic exposure and early postanoxic recovery in oyster tissues (Table 1 and Supplemental Table 1). In Cd-exposed oysters, L-alanine continued to accumulate in gills and muscle of control oysters reaching peak values after 1 h of reoxygenation (Table 1 and Supplemental Table 1). In Cd-exposed oysters, with significantly lower in Cd-exposed oysters compared with their control counterparts during both anoxia and early recovery; this difference was especially pronounced in muscle tissues.

Succinate and acetate, end products of mitochondrial anaerobiosis, also accumulated during prolonged anoxia, albeit at a slower rate than L-alanine (Table 1 and Supplemental Table 1). Succinate concentrations in gills and adductor muscle increased significantly above the normoxic levels only after 6 days of anoxia and returned to preexposure levels within 1 h of recovery. Dynamics of succinate levels during anoxia/recovery were similar in control and Cd-exposed oysters. Changes in acetate levels in gills and adductor muscle of control oysters followed similar dynamics to succinate, with a significant increase after 6 days of anoxia and return to the normoxic levels within 1 h of reoxygenation. In contrast, in Cd-exposed oysters no acetate accumulation was observed, and tissue acetate levels were significantly lower during anoxia in Cd-exposed oysters than in their control counterparts (Table 1 and Supplemental Table 1).

**High-energy phosphates and tissue energy status.** During normoxia, steady-state ATP levels were significantly lower in gills (but not in the muscle tissue) of Cd-exposed oysters.
compared with their control counterparts (Table 1 and Supplemental Table 1). ATP content significantly decreased during air exposure in tissues of control and Cd-exposed oysters, and returned to the preexposure levels during recovery (Table 1 and Supplemental Table 1). Recovery of the normoxic steady-state ATP levels took longer in Cd-exposed oysters compared with the controls, especially in the muscle (1 h vs. 6 h in control and Cd-exposed oysters, respectively). ADP levels remained fairly stable during anoxia and postanoxic recovery, whereas AMP significantly accumulated during air exposure, returning to the normoxic levels with 1 or 6 h of recovery (in control and Cd-exposed oysters, respectively). There was an overall depletion of adenylate levels during anoxic exposure, although it was only statistically significant in gills of control oysters and in adductor muscle of Cd-exposed ones (Table 1 and Supplemental Table 1). Total adenylate levels returned to the near-normoxic values during the 12 h recovery period in control and Cd-exposed oysters.

Normoxic levels of phospho-L-arginine (PLA) were higher in the adductor muscle compared with the gills and did not significantly differ between control and Cd-exposed oysters (Table 1 and Supplemental Table 1). PLA was significantly depleted during anoxia in the tissues of control oysters, while in Cd-exposed ones, PLA levels significantly rose during anoxic exposure reaching a peak after 1 h of postanoxic recovery. PLA levels returned to the normoxic, preexposure levels after 12 h of recovery in control and Cd-exposed oysters (Table 1 and Supplemental Table 1). There was no significant accumulation of l-arginine to mirror PLA depletion in control oysters, while in Cd-exposed oysters there was a decrease in l-arginine levels paralleling an increase in PLA concentrations during anoxia (Table 1 and Supplemental Table 1).

Oxidative DNA damage. Under normoxic conditions, the levels of DNA damage were comparable in control and Cd-exposed oysters (Fig. 6). Environmental anoxia and reoxygenation resulted in a significant accumulation of DNA lesions [as indicated by the mean number of apurinic/apyrimidinic (AP) sites in the total DNA] in Cd-exposed oysters but not in the control ones (Fig. 6).

DISCUSSION

Our study clearly demonstrates that intermittent oxygen deprivation strongly affects aerobic and anaerobic metabolism in hypoxia-tolerant intertidal mollusks, *C. virginica*. Metabolic responses to intermittent anoxia were significantly affected by exposure to Cd in oysters; therefore, for the sake of clarity we will first discuss the responses of control (not exposed to Cd) oysters and then compare them to their Cd-exposed counterparts.

As expected, the depletion of the hemolymph oxygen levels during air exposure resulted in transition to anaerobic metabolism shown by accumulation of anaerobic end products (L-alanine, acetate, and succinate). L-alanine was rapidly accumulated during early stages of anoxic exposure (especially in the muscle), followed by succinate and acetate at the later stages (by 3–6 days of air exposure), heralding an onset of deep mitochondrial anaerobiosis (42, 74, 75). Although prolonged anoxia resulted in a decrease of ATP levels in oysters (by 35–55% after 6 days of exposure), these changes were minimal (<10% per day) compared with hypoxia-sensitive organisms where ATP can be depleted within several minutes to hours of hypoxia (59, 74, 77). PLA levels did not significantly change during anoxia, indicating that anaerobic substrate-level phosphorylation along with the metabolic rate depression (40, 67) was sufficient to protect intracellular phosphagen pools in oysters.

Intermittent anoxia strongly affected mitochondrial function in oysters. Notably, mitochondrial responses of oysters to anoxia/reoxygenation stress demonstrated some similarities as well as important differences compared with hypoxia-sensitive organisms such as mammals. Prolonged air exposure resulted in a reduced maximum capacity of mitochondrial ETC in oysters (as indicated by a significant decline in the rate of uncoupled mitochondrial respiration by 17–25%). This finding agrees with earlier studies in mammalian models showing inhibitory effects of oxygen deficiency on activity of ETC complexes (including complexes I, II, and III) and matrix enzymes such as aconitase (32, 45, 55). In oysters, the anoxia-induced decrease in ETC capacity had no dramatic impact on ADP-stimulated or resting respiration rates during anoxia/reoxygenation. A slight (nonsignificant) trend for a decline in ADP-stimulated (state 3) respiration during anoxia was rapidly reverted upon return to normoxic conditions, making it unlikely that the anoxia-induced inhibition of ETC capacity is limiting, even under conditions of high metabolic fluxes during recovery. Notably, mitochondrial RCR significantly declined during anoxic exposure in oysters, reflecting the fact that mitochondrial proton leak stably remained at near-normoxic values throughout anoxia, while state 3 respiration declined, suggesting mild uncoupling. In contrast, phosphorylation efficiency (indicated by ADP/O ratios) was not affected for most substrates, except pyruvate. Anoxic exposure also had no effect on preferential oxidation of different substrates including fatty acids, amino acids, and glycolysis-linked substrates. The order of increasing RCR or ADP/O ratios for different substrates was also unaffected by prolonged anoxia or reoxygenation. This indicates that anoxia- and reoxygenation-induced changes in the relative abundance of metabolites that can be oxidized by mitochondria (e.g., L-alanine or succinate, Table 1 and Supplemental Table 1) have no effect on mitochondrial substrate preference. These data suggest that oyster mitochondria undergo minimal functional alterations during the prolonged “shutdown” in anoxic tissues, and the only significant functionally relevant change is a reduction in mitochondrial RCR.
A similar moderate decrease in RCR without a concomitant decline in oxidation capacity was found in mammalian mitochondria during hypoxia/reoxygenation-resistant states induced by ischemic preconditioning or by pharmacological agents and was interpreted as a sign of mild uncoupling that serves as a protection mechanism against elevated ROS formation and associated mitochondrial and tissue damage (44, 48, 52). Possibly a similar mechanism is involved in mitochondrial protection during postanoxic recovery in oysters.

In comparison, changes of mitochondrial properties during postanoxic reoxygenation in oysters were radically different from the hypoxia-sensitive organisms such as mammals. During the early period of reoxygenation (1–6 h), respiration rate of oyster mitochondria demonstrated a strong overshoot above the normoxic steady-state values. This postanoxic increase in oxygen consumption was especially pronounced in resting (state 4) mitochondria (−155–170%) compared with phosphorylating (state 3) mitochondria (−130–140%). Interestingly, a decrease in respiratory control ratio that was notable in mitochondria from anoxic oysters was not reverted by postanoxic recovery; in fact, RCR continued to decrease throughout 12 h of reoxygenation. This decrease was not associated with lower phosphorylation efficiency (measured as ADP/O ratios) and most likely reflected a stimulation of proton leak of resting mitochondria. There was no significant change in respiration rates in DNP-uncoupled mitochondria between normoxia and postanoxic recovery, suggesting that the observed overshoot is not due to an increase in the maximum capacity of ETC. Other possible mechanisms explaining elevated mitochondrial MO2 include a change in mitochondrial membrane potential, allosteric regulation of ETC activity, dephosphorylation of mitochondrial ETC complexes, elevated activities of substrate transport, and/or (in the case of state 3) increased activity of adenylate transport systems (32). Our current data does not allow determining which of these potential mechanisms are involved in the elevated mitochondrial MO2 during reoxygenation in oysters. However, regardless of the exact molecular mechanisms, this increase in oxygen consumption indicates elevated aerobic capacity of postanoxic mitochondria of oysters and may assist in the organism’s recovery after the period of oxygen depletion and associated energy deficiency. This finding is also interesting from the viewpoint of the much-debated mechanisms of so-called oxygen debt (an overshoot in whole organism oxygen consumption during postanoxic and posthypoxic recovery) in oysters and other hypoxia-tolerant organisms (20, 47, 72, 76). Although the causes of the oxygen debt are not fully understood, the current consensus attributes it to an overall increase in energy-demanding processes, such as metabolic costs of oxidation and excretion of anaerobic end products, resynthesis of phosphagen, ATP, and glycogen pools and restoration of acid base and ion homeostasis (47, 72, 73, 76). Our present study suggests that elevated mitochondrial proton leak may also contribute to the postanoxic oxygen debt in oysters; in the future, it would be interesting to determine whether this is broadly true for other hypoxia-tolerant organisms.

In hypoxia-sensitive mammalian models where mitochondrial events during hypoxia-reoxygenation have been extensively studied, the return to normoxia after a temporary oxygen deficiency (e.g., during ischemia/reperfusion) results in a strong free radical-mediated impairment of mitochondrial function, which occurs within the first few minutes of reoxygenation, involves a decrease in the rates of oxidative phosphorylation, loss of mitochondrial integrity (indicated by a dramatic decrease in RCR) and/or efficiency (indicated by lower ADP/O ratios) and sets the stage for tissue- and organ-wide functional injury (6, 35, 41, 52, 58, 78). This mitochondrial damage is associated with elevated generation of reactive oxygen (most notably, superoxide, O2−) and nitrogen (especially peroxynitrite, ONOO−) species during reoxygenation (1, 37, 41, 78). In stark contrast to these findings, there was a significant increase of respiratory rates of oyster mitochondria during postanoxic recovery going hand-in-hand with the rapid restoration of steady-state ATP levels. Notably, accumulation of l-alanine also continued during the early postanoxic recovery (1 h), suggesting that anaerobic pathways supplement aerobic ATP production during reoxygenation. No sign of oxidative damage (at least at the DNA level) was detected during anoxia/reoxygenation in control oysters, suggesting that unlike in mammals, mitochondrial ROS production either does not increase during anoxia-reoxygenation or is efficiently counteracted by antioxidant systems in oyster tissues. It is likely that this remarkable resistance of oyster mitochondria to reoxygenation-induced injury is at the root of their high tolerance to periodic oxygen deprivation and reflects adaptation to the life in the intertidal zone where frequent fluctuations in oxygen availability are common.

Our study also shows that exposure to additional environmental stressors (such as Cd) can significantly alter metabolic responses to intermittent anoxia in oysters, thus potentially affecting their ability to survive periodic oxygen deprivation. Indeed, Cd exposure had a significant effect on both aerobic and anaerobic metabolism of oysters during anoxia and reoxygenation. Accumulation rates of anaerobic end products (l-alanine and acetate) were significantly lower in Cd-exposed oysters compared with their control counterparts, and no overshoot in l-alanine levels was observed during the first hour of recovery in Cd-exposed oysters. This indicates that at least some key anaerobic metabolic pathways are inhibited by Cd exposure in oysters. Despite this inhibition, a decrease in ATP levels during prolonged air exposure was similar in control and Cd-exposed oysters. Interestingly, phosphagen levels slightly but significantly increased during anoxia in Cd-exposed oysters. This suggests that Cd may affect the steady state of arginine kinase reaction in such a way that PLA is not used up and even accumulates despite the depletion of ATP levels. The physiological consequences of the latter change are not known, and while they may not be important during metabolic rate depression (e.g., in anoxia) when PLA use is slow or absent, they may come into play under conditions of high metabolic fluxes when rapid PLA breakdown is required to buffer intracellular ATP levels, such as during reproduction or temperature stress (27, 62, 64, 65).

Cd exposure strongly affected mitochondrial responses to postanoxic recovery in oysters by completely abolishing an overshoot in mitochondrial capacity during reoxygenation. Moreover, unlike in the controls, in Cd-exposed oysters there was a steady decline in respiration rate of phosphorylating (state 3) mitochondria during postanoxic recovery, indicating mitochondrial deterioration and going hand-in-hand with a progressive loss of mitochondrial integrity. Notably, unlike the control oysters, there was no overshoot in the hemolymph PO2...
during postanoxic recovery in Cd-exposed mollusks, suggesting that Cd exposure prevented postanoxic hyperventilation to pay off the oxygen debt. This, along with the disturbance of mitochondrial phosphorylation capacity, may explain the delayed recovery of steady-state ATP levels in Cd-exposed oysters, especially in muscle tissues. Inhibition of mitochondrial function by prolonged anoxia and subsequent recovery in the presence of Cd was concurrent with elevated levels of oxidative DNA lesions in Cd-exposed oysters. These changes are reminiscent of the typical mitochondrial alterations induced by intermittent oxygen deficiency in hypoxia-sensitive organisms, such as mammals (1, 29, 37, 52, 77), and suggest that Cd exposure may lead to the loss of the stress-resistant mitochondrial phenotype in oysters. Earlier studies showed that Cd can inhibit mitochondrial ETC and matrix enzymes and induce elevated rates of ROS generation in oysters (12, 33). These pro-oxidant effects of Cd may augment reoxygenation-induced ROS production and explain mitochondrial deterioration and oxidative DNA damage observed during combined exposure to Cd and anoxia-reoxygenation stress in this study. Another possible explanation for the higher frequency of DNA lesions in Cd-exposed oysters is inhibition of DNA repair machinery by Cd (15, 25). Specifically, apurinic/apyrimidinic (AP) endonuclease 1, which initiates repair of AP lesions by incising the DNA backbone, is inhibited by Cd$^{2+}$ at high concentrations (>10 μM) (50). These two mechanisms (elevated ROS production and inhibited DNA repair) are not mutually exclusive and both likely contribute to the accumulation of AP lesions in DNA of Cd-exposed oysters during anoxia and reoxygenation.

Perspectives and Significance

Mitochondrial adaptations to intermittent anoxia in a hypoxia-tolerant mollusk C. virginica involve mild uncoupling and an overshoot of mitochondrial respiration during postanoxic recovery. This dramatically differs from rapid mitochondrial deterioration induced by postanoxic reoxygenation in hypoxia-sensitive organisms such as mammals. Anaerobic metabolism combined with the metabolic rate depression ensures long-term anoxic survival of oysters, and anaerobic L-alanine production also appears to play a role during early aerobic recovery, likely augmenting the rapid restoration of the steady-state ATP levels. Earlier studies in mammals demonstrated that substrate-level anaerobic phosphorylation can also render protection against mitochondrial damage during hypoxia-reoxygenation stress (74, 75). This raises an intriguing possibility that anaerobic metabolic pathways may play a dual role for ATP synthesis and mitochondrial protection during intermittent oxygen deficiency in oysters and warrants further investigation. Exposure to a toxic metal, Cd, abolishes reoxygenation-induced stimulation of mitochondrial respiration and leads to oxidative stress and a loss of mitochondrial capacity during postanoxic recovery. Considerable accumulation of oxidative DNA lesions during anoxia and reoxygenation in Cd-exposed oysters can contribute to mitochondrial dysfunction via mitochondrial DNA damage and may also increase the risk for reduced reproductive fitness in oysters. This would result in increased sensitivity to intermittent hypoxia and anoxia in Cd-exposed populations of the mollusks and will have implications for survival of oysters in estuaries and coastal zones where the increasing expanse of dead zones is combined with anthropogenic pollution.

ACKNOWLEDGMENTS

The authors thank Dr. Cheryl M. Woodley of National Oceanic and Atmospheric Administration (NOAA) Center for Coastal Environmental Health and Biomedical Research (CCEHBR), Charleston, SC for technical assistance and helpful suggestions, and the three anonymous reviewers for their useful comments on the earlier version of the manuscript.

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GRANTS

This work was supported by funds provided the National Science Foundation CAREER award (IBN-0347238), and a University of North Carolina, Charlotte, ADVANCE program Bonnie Cone Fellowship (through a National Science Foundation ADVANCE Institutional Transformation Program Grant NSF-0548401) to I. M. Sokolova. L. A. May was supported by NOAA CCEHBR, NOAA’s Coral Reef Conservation Program via the Coral Disease and Health Consortium.

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