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Effects of hypoxia on gene and protein expression in the blue crab, *Callinectes sapidus*

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Abstract

Increases in hypoxic conditions are one of the major factors responsible for declines in estuarine habitat quality, yet to date there are no indicators for recognizing populations of estuarine organisms that are suffering from chronic hypoxic stress. Here we test the hypothesis that alterations in gene and protein expression of antioxidant enzymes and other stress-specific proteins can be used as molecular indicators of hypoxic stress. Blue crabs, *Callinectes sapidus*, were exposed to 2–3 ppm DO for 5 days. Gene expression was measured using macroarrays constructed from cDNA of 10 partial gene transcripts cloned from blue crab hepatopancreas. Significant ($p \leqslant 0.05$) down-regulation of gene expression was found for MnSOD, hemocyanin, ribosomal S15 and L23. Subtractive hybridization using RNA from control and hypoxic hepatopancreas tissues also indicated down-regulation of hemocyanin transcription. In contrast, Western blotting showed a significant ($p \leqslant 0.05$) increase of hemocyanin protein in the hepatopancreas and cross-linking of MnSOD proteins in hypoxia-exposed crabs. Thus, hypoxia-responsive cDNA arrays and Westerns may be useful diagnostic tools for monitoring effects of hypoxia in estuarine crustacea.

Keywords: Hypoxic stress; cDNA array; Hemocyanin; MnSOD; Molecular biomarkers

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Hypoxic conditions in estuarine ecosystems are increasing in frequency, intensity, duration and extent,and are responsible for serious declines in habitat quality. Oxygen depletion events have occurred in 32 of 38 Gulf of Mexico estuaries (Bricker, 1997), and there is a well-known expansive area of seasonal hypoxia/anoxia on the Louisiana continental shelf (Turner & Rabalais, 1994). The effects of hypoxia on biota are often inferred from measurements of low oxygen levels which coincide with catastrophic mortality of various organisms (Winn & Knott, 1992). Thus, indicators at the organismal/cellular level are needed that can be used to assess the onset, duration and severity of hypoxia and its effect on biota. We hypothesize that alterations in protein concentration and gene expression of antioxidant enzymes and other stress-specific proteins can be used as molecular indicators of hypoxic stress. Here, we examine the effects of low oxygen on the blue crab, *Callinectes sapidus*, an estuarine organism that frequently encounters hypoxic stress.

Five adult blue crabs in 35 L replicate aquaria were exposed to hypoxia (2–3 mg/L DO) for 5 days in filtered seawater adjusted to a salinity of 15 g/L at 27 °C in an intermittent flow-through system (Manning et al., 1999). Normoxic controls were held at 6–8 mg/L DO. Flow rate was sufficient to provide approximately 4.0 volume additions/day (126 cycles per day of 1 L each cycle) in each treatment aquarium. To achieve the desired DO of 2–3 ppm, oxygen in the flow-through dilution water was maintained in a covered headbox at supersaturation (14 ppm). This high DO concentration was necessary because of the high rates of oxygen consumption by the study crabs. The normoxic dilution water was maintained from 18 to 20 ppm before delivery to normoxia aquaria.

Hepatopancreatic tissues from 10 crabs in each treatment were analyzed by Western blots for stress proteins and RNA was extracted for analysis of gene expression using cDNA arrays. Ten genes cloned and sequenced from hepatopancreatic tissue of wild-caught blue crabs (metallothionein [two isoforms], MnSOD [two isoforms], Heat Shock Protein 70, hemocyanin, actin and ribosomal proteins S15, S20 and L23) were PCR amplified, spotted in duplicate onto neutral nylon macroarrays and hybridized as previously described (Larkin, Folmar, Hemmer, Poston, & Denslow, 2003). For each cDNA clone, the general background of each membrane was subtracted from the average value of the duplicate spots on the membrane. The values were normalized to the average value of a β-actin cDNA clone. For the subtractive hybridizations (Clontech, Palo Alto, CA), mRNA samples from hepatopancreas of normoxic and hypoxic blue crabs were reverse transcribed into cDNA, heat denatured and the cDNA pools were then hybridized together. For cloning of hypoxia-upregulated genes "normoxic cDNA" was used as the driver, whereas for down-regulated genes "hypoxic cDNA" served as the driver. The cDNAs that remained un-hybridized, which represent putative differentially regulated mRNAs, were then PCR amplified, cloned and sequenced for identification.

Significant decreases (Student's T-test, $p < 0.05$) in transcription of one of the MnSOD isoforms, hemocyanin and ribosomal S15 and L23 genes were observed in hypoxia-exposed crabs. The decrease in two of the three ribosomal cDNAs analyzed suggests that protein synthesis may be starting to shut down in these animals. Interestingly, while there were no significant differences between hypoxic and normoxic

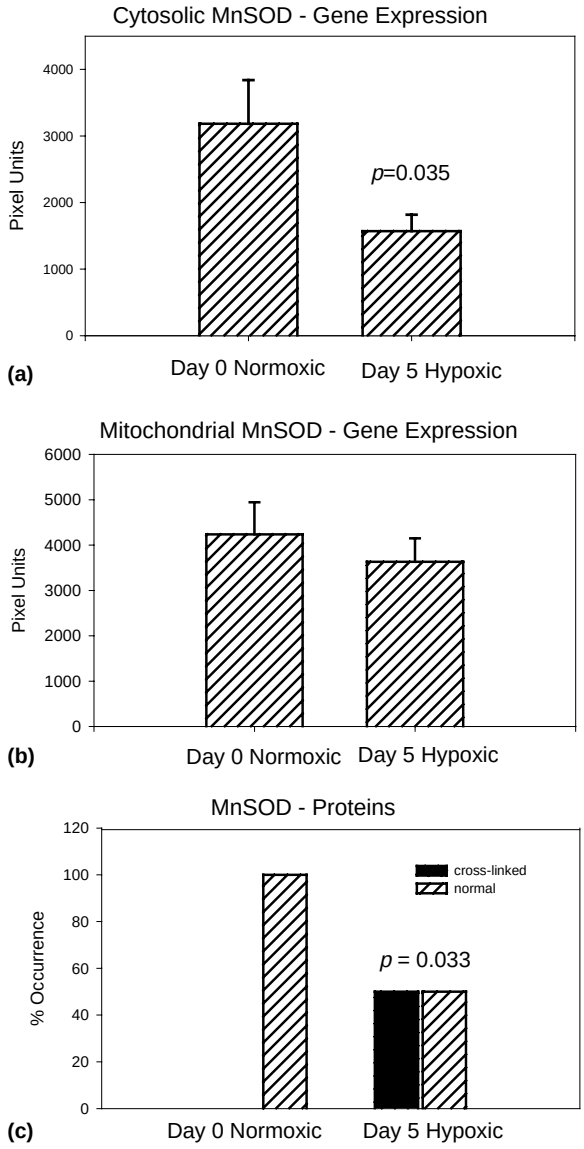

Fig. 1. Results of gene and protein expression of MnSOD in normoxic and hypoxic blue crabs. Gene expression of cytosolic MnSOD (a) and mitochondrial MnSOD (b) was determined from macroarrays. Bars represent mean ± SE of nine individuals in each group. Pixel units refer to the intensity of the radiolabeled spots on the macroarrays normalized to the intensity of β actin. Protein expression of both isoforms of MnSOD (c) was determined with Western Blot analysis, using an anti-MnSOD antibody (SOD-111) from Stressgen and chemiluminescence detection. The graph shows the percentage of crabs with normal or cross-linked MnSOD.

crabs for the other genes tested, a general trend of down-regulation of gene expression after 5 days hypoxia was observed (data not shown).

Blue crabs lack Cu, ZnSOD and instead have two MnSOD isoforms, one in the cytosolic compartment and one in the mitochondria (Brouwer, Hoexum-Brouwer, Grater, Enghild, & Thogersen, 1997). Gene transcription of the mitochondrial isoform was significantly down-regulated in response to hypoxia ($p = 0.035$; Fig. 1(a)), but transcription of cytosolic MnSOD was not affected ($p = 0.50$;

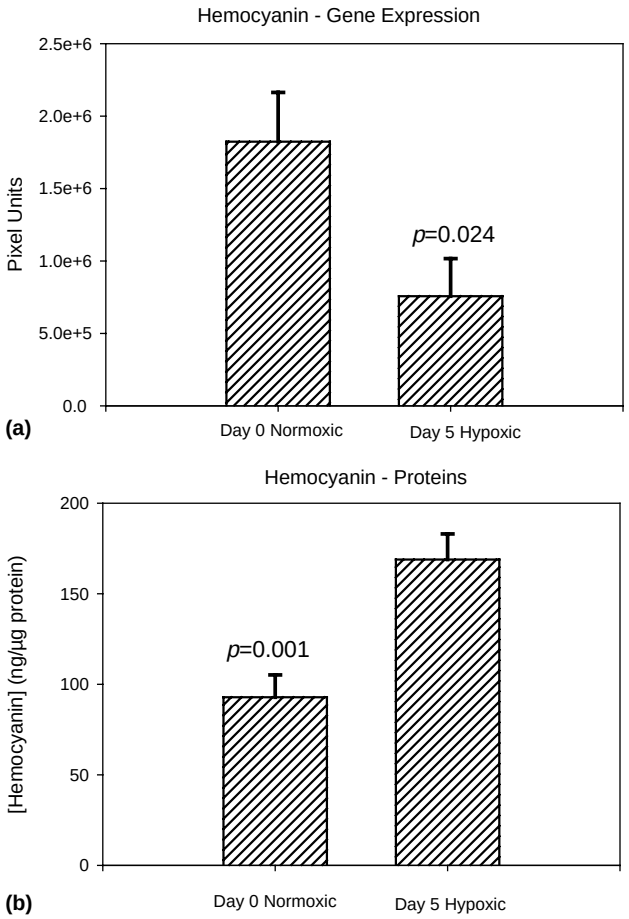

Fig. 2. Results of gene and protein expression of hemocyanin in normoxic and hypoxic blue crabs. Gene expression of hemocyanin (a) was determined from macroarrays. Bars represent mean ± SE of nine individuals in each group. Pixel units refer to the intensity of the radiolabeled spots on the macroarrays normalized to the intensity of β actin. Hemocyanin concentration in hepatopancreas (b) was determined with Western Blot analysis of hepatopancreas extracts and purified hemocyanin standards using a rabbit anti-blue crab hemocyanin polyclonal antibody (Duke University Vivarium) and chemiluminescence detection. Total protein was determined with the BCA (Pierce) Protein Assay. Bars represent mean ± SE of 10 individuals in each group.

Fig. 1(b)). The SOD proteins showed a dramatic effect of hypoxia (Fig. 1(c)), with an unexpected, and as yet unexplained, cross-linking of MnSOD proteins (subunit MW ~22 kDa) into higher molecular weight (~70–90 kDa) aggregates. Cross-linking of MnSOD was not seen in the normoxic controls, but there was a significant increase (Fisher's Exact Test, $p = 0.033$) in high-molecular weight MnSOD after 5 days exposure to hypoxia. The potential functional significance of this phenomenon is unknown.

Hemocyanin gene expression was significantly ($p = 0.024$) down-regulated after a 5 days hypoxic exposure (Fig. 2(a)). In contrast, hemocyanin protein concentrations significantly increased ($p = 0.001$; Fig. 2(b)). The down-regulation of hemocyanin mRNA suggests the crabs are shutting down aerobic metabolism after 5 days exposure to hypoxia. The increased levels of protein still present in the hepatopancreas suggest that turnover of hemocyanin protein is slow. A shorter-term experiment might show upregulation of the hemocyanin gene as an immediate response to hypoxia, followed by the down-regulation observed here.

Results of the subtractive hybridization support data obtained from the macroarrays, in that hemocyanin as well as cryptocyanin (a copperless hemocyanin homologue) appear to be down-regulated in 5 days hypoxic crabs. In addition, it appears that transcription of cytochrome *c* oxidase subunit 1 is also down-regulated, a further indication that the aerobic metabolic pathway may be shutting down. The genes obtained through the subtractive hybridization will be spotted on macroarrays, permitting more conclusive results regarding the effects of hypoxia on their regulation.

The results to date are encouraging in the search for molecular indicators of hypoxia. However, data presented here represent only one single point in time, and both shorter and longer-term exposures to hypoxia are necessary to establish the temporal changes in gene expression and protein synthesis in response to hypoxia. Finally, the observation that hemocyanin mRNA decreases whereas hemocyanin protein increases stresses the importance of verifying if gene expression patterns truly reflect protein responses.

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