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Hypoxia-modulated gene expression profiling in sea urchin (*Strongylocentrotus nudus*) immune cells

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ABSTRACT

Hypoxia is an issue that affects ocean coastal waters worldwide. It has severe consequences for marine organisms, including death and rapid adaptive changes in metabolic organization. Although some aquatic animals are routinely exposed and resistant to severe environmental hypoxia, others such as sea urchins (*Strongylocentrotus nudus*) have a limited capacity to withstand this stress. In this study, hypoxia induced a significant increase in the number of red spherule cells among coelomocytes, which function as immune cells. This suggests that sea urchin immune cells could be used as a biological indicator of hypoxic stress. In the current study, we used cDNA microarrays to investigate the differential expression patterns of hypoxia-regulated genes to better understand the molecular mechanisms underlying the response of immune cells to hypoxia. Surprisingly, the predominant major effect of hypoxia was the widespread suppression of gene expression. In particular, the expression of RNA helicase and GATA-4/5/6 was decreased significantly in response to hypoxia, even in field conditions, suggesting that they could be utilized as sensitive bioindicators of hypoxic stress in the sea urchin.

1. Introduction

Rapid changes in dissolved oxygen (DO) levels in coastal waters have occurred over recent decades. The combined effects of the continued spread of coastal eutrophication and global warming have led to the widespread occurrence of hypoxia, a condition in which DO is below the level necessary to sustain organismal life, defined as <2 mg/l (Vaquer-Sunyer and Duarte, 2008). The area of oceanic hypoxic zones has increased continuously since 1960, with a current total area of $\sim$245,000 square kilometers worldwide (Diaz and Rosenberg, 2008). This has caused severe changes in marine ecosystems in the adjacent waters, which has resulted in the mass death of organisms including fishes, echinoderms, crustaceans, mollusks, and cnidarians (Vaquer-Sunyer and Duarte, 2008).

Recently, molecular studies were performed to investigate the effects of hypoxia on aquatic organisms, including invertebrates and fish. Data revealed that the hypoxic response upregulated genes related to glycolysis, iron metabolism, amino acid metabolism and growth suppression, whereas genes related to translation were downregulated in aquatic organisms (Gracey et al., 2001; Padilla and Roth, 2001; Holm et al., 2008). In addition, a decrease in the expression of the GTP-binding protein Rab, which is relevant to cell growth and proliferation, and increases in HSP70 and glutathione reductase (GR) levels were observed in the embryos of zebrafish and sea stars exposed to hypoxia (Padilla and Roth, 2001; Holm et al., 2008). The consumption of green sea urchins was found to be decreased dramatically in oxygen-depleted environments, suggesting altered expression patterns of genes associated with translation (Morales et al., 2006; Siikavuopio et al., 2007). Nevertheless, limited studies have assessed the molecular aspects of the responses of marine organisms, including sea urchins, to hypoxia.

Because they are suitable biological organisms for investigating the quality of seawater, sea urchins are becoming widely used to assess seawater pollution and the effects of marine environmental changes on aquatic organisms. For example, genome studies in purple sea urchins were performed recently (Hood and Fernandez, 2008; Hibino et al., 2006). *Strongylocentrotus nudus* belongs to the family Strongylocentrotidae and mostly exists on the coasts of South Korea. *S. nudus* is advantageous as a model organism for studies assessing the initial effects of external stresses, including hypoxia (Ryu et al., 2012). For example, coelomocytes were

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recognized as immune cells because of their ability to respond to various environmental stresses, such as temperature and UV-radiation (Matranga et al., 2005, 2006). The number of red spherule cells, a minor immune cell group, was increased in sea urchins exposed to different physical and chemical stresses (Glinski and Jarosz, 2000; Matranga et al., 2000), suggesting that sea urchin immune cells might be novel cellular biosensors of environmental stress (Pinsino et al., 2008).

However, the molecular mechanisms underlying the response of immune cells to hypoxia in sea urchins are not fully understood. The aim of this study was to investigate the number of red cells and cell death in the immune cells of the sea urchin in response to hypoxia. cDNA microarray analysis was also performed to identify the genes differentially expressed in sea urchin immune cells under hypoxic conditions. Data from both the laboratory and the field revealed that RNA helicase and GATA-4/5/6 were hypoxia-repressed genes, suggesting they might act as biomarkers of the stress response to hypoxia in sea urchins.

2. Materials and methods

2.1. Sea urchin culture

Strongylocentrotus nudus used in this study were collected directly from the coast of Geoje, South Korea, and were cultured at 18 °C, pH 8.45 and 33.72 (‰), and fed seaweed in the culturing room of the Korea Institute of Ocean Science and Technology. The diameter of the sea urchins used in this study was ~7–10 cm. Hypoxic (DO=1.6 mg O_2/L), normal (DO=7.6 mg O_2/L), and intermediate (DO=4.7 mg O_2/L) environments were maintained using an aquacontroller (Neptune systems, Water Management Technologies Inc., USA). The levels of DO were measured using an oxygen probe with Unidata Starlog data loggers (cat# 7422A, Unidata Pty. Ltd., Australia) every 6 h. Adult sea urchins were incubated for 0, 6, 12, or 24 h in a 50-L water bath filled with filtered sea water (FSW). Twenty-four sea urchins were used in each treatment, with six harvested at per time point (0 h, 6 h, 12 h, 24 h). In the field, chambers containing fifteen sea urchins and 100 g seaweed were settled in normoxic (1 m) and hypoxic regions (9 m). After settling, sea urchins were cultured for seven days and then harvested. Environmental factors such as DO, temperature, salinity, and pH were measured daily.

2.2. Color change in sea urchin immune cells

After sampling, the peristomial membrane around the mouth of the sea urchin was removed using scissors, and the immune cells were collected using 50-mL syringes. The collected immune cells were divided among the wells of a 12-well plate, and the colors of the immune cells in each treatment condition were compared.

2.3. Cell death assay

Hypoxia-induced cell death was measured using a LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen, CA, USA). Sea urchins were exposed to hypoxia (DO=1.6 mg O_2/L) and normoxia (DO=7.6 mg O_2/L) for 0, 6, 12, and 24 h in a 50-L water bath. Immune cells (~10^6 cells) were centrifuged at 1000*g* for 5 min and then resuspended in 200 μL PBS. Next, 5 μL 2 mM EthD-1 and 3 μL 4 mM calcein AM were added, after which the cells were vortexed and incubated at room temperature for 30 min. The immune cells were then washed with D-PBS and observed under the UV filter of a fluorescent microscope (Carl Zeiss, Axioplan II, Welwyn Garden, Germany). Viable and non-viable cells were colored green and red, respectively.

2.4. Microarrays

Expression profiling was performed using the *Strongylocentrotus purpuratus* 135k microarray (NimbleGen Inc; http://www.nimblegen.com/). The 135k microarray was designed from 89,602 UniGenes clustered from 141,833 expressed sequence tags (ESTs) and 44,561 cDNAs available at NCBI (http://www.ncbi.nlm.nih.gov/). Three 60-nucleotide (nt)-long probes were designed along each gene, starting 60 bp prior to the stop codon and in 30-bp increments, such that the three probes covered 120 bp of the 3′ region of the gene. A total of 133,244 probes were designed, with a mean probe size of 60 nt and melting temperatures of 75–85 °C. Selection markers including *gfp*, *gus*, *hyg*, *bar*, and *kan* were also included. Random GC probes (38,000) were included to monitor hybridization efficiency, and four corner fiducial controls were included to assist with overlaying the grid on the images. The microarray analyses were repeated three times. A RevertAid H (−) first strand cDNA synthesis kit (Fermentas, Ontario, Canada) was used to synthesize double-stranded DNA, and the synthesized cDNA was cleaned utilizing a MinElute reaction kit (Fermentas). For microarray hybridization, 10 μg DNA was mixed with 19.5 μL 2× hybridization buffer and 39 μL distilled water (DW). Hybridization was performed in a MAUI chamber at 42 °C for 18 h. After hybridization, the samples were washed twice and scanned using a GenePix Scanner 4000B (Axon, CA, USA).

2.5. Quantitative real-time PCR

Total RNA was isolated from the immune cells of sea urchins, and cDNA synthesis was performed using the Reverse Transcription System (Promega, Madison, WI, USA). Actin was used as the housekeeping gene, and quantitative real-time PCR (qRT-PCR) was performed to assess the expression of six different genes identified in the microarray analyses. The primers used in qRT-PCR are summarized in Table S1. Differences in gene expression were quantified using the delta CT method. The qRT-PCR reactions were performed using the following conditions: 94 °C for 5 min, followed by 40 cycles at 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 15 s. A reaction volume of 20 μL was used for the PCR amplifications, and the reactions were performed by mixing 2 μL 10× reaction buffer, 2 μL 2.5 mM dNTPs, 0.5 μL Taq DNA polymerase, 5 μL cDNA, and 1 μL SYBR.

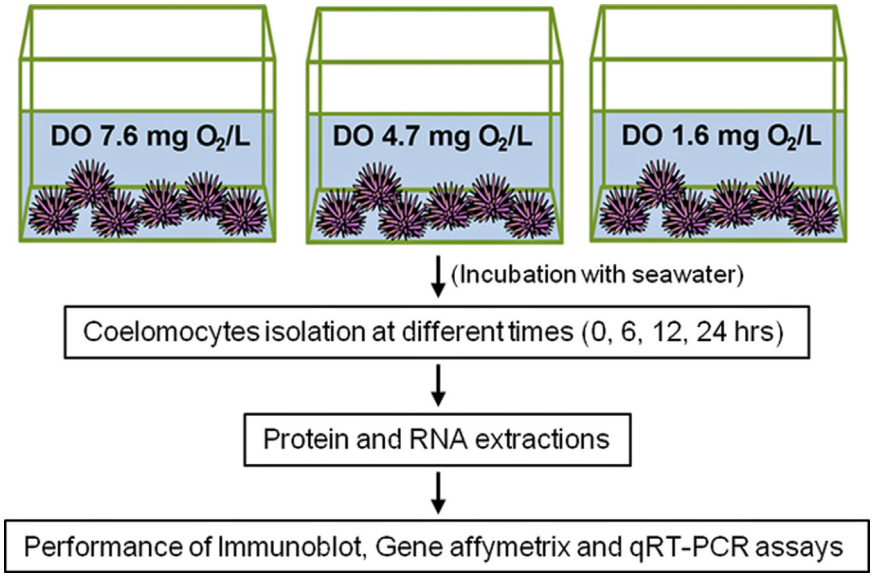

Fig. 1. A schematic diagram to show how to prepare the sea urchin coelmocytes.