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Gene expression of apoptosis-related genes, stress protein and antioxidant enzymes in hemocytes of white shrimp *Litopenaeus vannamei* under nitrite stress

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ABSTRACT

Apoptotic cell ratio and mRNA expression of caspase-3, cathepsin B (CTSB), heat shock protein 70 (HSP70), manganese superoxide dismutase (MnSOD), catalase (CAT), glutathione peroxidase (GPx) and thioredoxin (TRx) in hemocytes of white shrimp *Litopenaeus vannamei* exposed to nitrite-N (20 mg/L) was investigated at different stress time (0, 4, 8, 12, 24, 48 and 72 h). The apoptotic cell ratio and mRNA expression level of CTSB were significantly increased in shrimp exposed to nitrite-N for 48 and 72 h. Caspase-3 mRNA expression level significantly increased by 766.50% and 1811.16% for 24 and 48 h exposure, respectively. HSP70 expression level significantly increased at 8 and 72 h exposure. MnSOD mRNA expression in hemocytes up-regulated at 8 and 48 h, while CAT mRNA expression level increased at 24 and 48 h. GPx expression showed a trend that increased first and then decreased. Significant increases of GPx expression were observed at 8 and 12 h exposure. Expression level of TRx reached its highest level after 48 h exposure. These results suggest that nitrite exposure induces expression of apoptosis-related genes in hemocytes, and subsequently caused hemocyte apoptosis. Meanwhile, expression levels of HSP70 and antioxidant enzymes up-regulated to protect the hemocyte against nitrite stress.

1. Introduction

Nitrite is formed from ammonia by the nitrification process and may be accumulated in aquatic systems as a result of imbalances of nitrobacteria activity, *Nitrosomonas* sp. and *Nitrobacter* sp. (Mevel and Chamroux, 1981). It is one of the most common pollutants in intensified aquaculture or in recirculated water. It has been reported that the concentration of nitrite increases directly with culture period, and might reach as high as 20 mg/L in grow-out ponds of white shrimp *Litopenaeus vannamei* (Tacon et al., 2002).

It is well known that aquatic animals are more at risk towards nitrite intoxication because nitrite can be take up across gill epithelium and accumulated to very high concentrations in the body fluids (Jensen, 2003). Accumulation of nitrite in pond water may deteriorate water quality, retard growth and molting, increase oxygen consumption and ammonia excretion, and even cause high mortality of shrimp (Chen and Chen, 1992; 1995a, 2001). In crustaceans it has been also suggested that elevated concentration of nitrite in pond water influences hemocyanin formation, causes hypoxia in tissues, impairs the respiratory metabolism, depresses immunologic function, increases susceptibility to bacterial infection (Chen and Cheng, 1995b; Tseng and Chen, 2004; Wang et al., 2004; Chand and Sahoo, 2006). However, the molecular mechanism of nitrite stress-induced injury remains unclear.

Pervious study demonstrated that oxidative stress was one of the toxicity mechanisms of nitrite on shrimp (Xian et al., 2011). Antioxidant enzyme systems are a well-developed regulatory mechanism protecting against oxidative stress. Under normal physiological states, reactive oxygen species (ROS) are rapidly eliminated by antioxidant enzymes, including superoxide dismutase (MnSOD), catalase (CAT), glutathione peroxidase (GPx) and thioredoxin (TRx). Published researches had showed that gene expression of antioxidant enzymes in shrimp would be affected by some environmental factors, such as pH (Wang et al., 2009) and temperature (Zhou et al., 2010). However, the relationship between nitrite stress and gene expression of antioxidant enzymes remains unknown largely.

ROS overproduction paralleled hemocyte apoptosis were observed in shrimp exposed to some environmental stress factors, such as Cu^{2+} (Xian et al., 2010) and nitrite (Xian et al., 2011). Apoptosis is a normal physiological process for removal of excess, damaged, necrotic, or potentially dangerous cells such as virus-infected cells (Xian et al., 2013). Caspase activation plays a central role in the execution of programmed cell death (Porter and Janicke, 1999). Full-length

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caspase-3 complementary (c) DNA had been cloned and characterized in some species of shrimp (Wongprasert et al., 2007; Chang et al., 2008). It was reported that caspase-3 play an important role in hemocyte apoptosis of shrimp under pathogen infection (Wongprasert et al., 2007; Chang et al., 2008; Rijiravanich et al., 2008) and low temperature stress (Chang et al., 2009).

Cathepsin B (CTSB) which locates in lysosome is a cysteine proteinase scarcely studied in crustaceans (Stephens et al., 2012). It was demonstrated that these lysosomal enzymes participates hydrolyze protein intracellularly, as its common function in vertebrates (Stephens et al., 2012). Other studies suggested that cathepsin family might contribute to the degradation of both viral and bacterial materials (Robalino et al., 2007; Pongsomboon et al., 2008). Additionally, cathepsins have been reported to be involved in apoptosis (Chwieralski et al., 2006; Liu et al., 2006). However, the role of cathepsins in apoptosis has not been clearly described. In the present study, role of caspase-3 and CTSB in nitrite-induced hemocyte apoptosis of shrimp was investigated.

Under conditions of stress, heat shock proteins (HSPs) act as molecular chaperones to regulate protein homeostasis and to prevent aggregation and to assist refolding of misfolded proteins. The expression of HSP has been reported to be induced by various stress conditions, such as heat shock (Rungrassamee et al., 2010; Zhou et al., 2010), pathogen infection (Rungrassamee et al., 2010; Huang et al., 2011) and heavy metals exposure (Qian et al., 2012). Effect of nitrite on HSP gene expression in shrimp has not been studied.

The aim of our study is to investigate changes occurring in the gene expression of apoptotsis-related protein (caspase-3, CTSB), stress protein (HSP70) and antioxidant enzymes (MnSOD, CAT, GPx and TRx) in hemocytes of *L. vannamei* under nitrite stress. Hemocyte apoptosis in nitrite-exposed shrimp was also determined by flow cytometry.

2. Materials and methods

2.1. Animals

The experiment shrimp *L. vannamei* (4.41 ± 1.80 g) were obtained from a commercial farm in Panyu (Guangdong, China), and acclimated for 2 weeks prior to experiments in cycling-filtered plastic tanks with aerated seawater at 26 ± 2 °C and a salinity of 5‰. During the acclimation period, shrimp were fed twice daily with shrimp diet (40% protein, 5.0% fat, 5.0% fiber and 16% ash, supplied by a commercial diet, China) until 24 h before the experimental treatments began. Only shrimp apparently healthy and in the intermolt stage were used for the study. The molt stage was determined by examining the uropoda in which partial retraction of the epidermis could be distinguished (Robertson et al., 2007).

2.2. Nitrite exposure

Exposure experiment was conducted in triplicate with twenty-five shrimps in plastic tank with 180 L water (26 ± 2 °C, pH 7.9–8.0, salinity 5‰). Each tank was aerated continuously using an aeration stone. Two concentrations of nitrite-N (zero control and 20 mg L^{-1}) were set in the exposure experiment. The nitrite solution was prepared by adding $NaNO_2$ to 5‰ seawater until the desired concentration was attained.

2.3. Hemolymph samples

At the beginning and after 4, 8, 12, 24, 48 and 72 h of exposure, three shrimp were randomly sampled from each tank. Hemolymph (200 μL) was withdrawn from the ventral sinus of each shrimp by a 1 mL sterile syringe (25 gauge) containing an equal volume of ice-cold anticoagulant (glucose 20.5 g L^{-1}, sodium citrate 8 g L^{-1}, sodium chloride 4.2 g L^{-1}, pH 7.5). The hemolymph from each shrimp was transferred into an individual tube held on ice. Fifty microliters of hemolymph were removed for hemocyte apoptosis analysis. The remaining hemolymph was centrifuged at 800 *g* for 10 min at 4 °C, and then the cell pellets were resuspended in Trizol (Invitrogen) and stored at −80 °C for gene expression analysis.

2.4. Apoptotic cell ratio

The apoptotic hemocytes were examined using Annexin V-FITC/PI apoptosis detection kit (Invitrogen). Annexin V-FITC (fluorescein isothiocyanate) is used to quantitatively determine the percentage of cells that are actively undergoing apoptosis. PI (Propidium iodide) binds to double-stranded DNA and fluoresces at wavelengths above 630 nm, it enters and stains nonviable cells but cannot cross the membrane of viable cells, thereby PI is regarded as a viability probe and used to distinguish viable from nonviable cells (Hégaret et al., 2003). Fifty microliters of hemolymph was diluted with anticoagulant to obtain a final concentration of 1×10^6 cells mL^{-1}. Hemocyte suspension (300 μL) was centrifuged and resuspended at about 3×10^6 cells mL^{-1} in 1 × Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). A 100 μL hemocyte sample was stained with 5 μL of Annexin V-FITC and 10 μL of 50 μg mL^{-1} PI working solution for 15 min in the dark. Then 400 μL 1 × Annexin V binding buffer was added to each tube, and the cells were immediately analyzed by flow cytometry. For each sample, 10,000 events were counted. Results were expressed as Annexin V-FITC/PI dot plot. Cells stained negative with both probes represent live cells. Cells stained positive with Annexin V-FITC and negative with PI are early apoptotic cells. Cells stained positive with both Annexin V-FITC and PI are in the end stage of apoptosis, undergoing necrosis, or already dead. The apoptotic cell ratio (AR) defined as the percentage of apoptotic hemocytes, was expressed as:

$$AR = (\text{early apoptotichemocytes} + \text{late apoptotic and necrotichemocytes}) / (\text{total hemocytes}) \times 100\%.$$

2.5. Gene expression in hemocytes

2.5.1. Total RNA extraction and cDNA synthesis

Total RNA of hemocytes was extracted using Trizol method (Invitrogen, USA) according to the manufacturer's instructions. The quantity and quality of each RNA were assessed by measuring their absorbance at 260 and 280 nm using an NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA), and RNA integrity was verified on agarose electrophoresis. First-strand cDNA was synthesized with total RNA 1 μg in each reaction systems using PrimeScript RT reagent Kit With gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocol. All cDNAs were diluted to 50 ng μL^{-1} with nuclease-free water and stored at −20 °C until used as templates in real-time quantitative PCR (qRT-PCR).

2.5.2. Real-time qRT-PCR assay

Specific primer pairs were designed based on published *L. vannamei* mRNA sequences available in NCBI using Primer Premier v5 (PREMIER Biosoft International, Palo Alto, CA, USA) or according to previous descriptions as shown in Table 1. Real-time qRT-PCR was carried out in an ABI 7500 real-time PCR machine (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara, Dalian, China) following the manufacturer's recommendations. The reaction mixtures were in a volume of 20 μL containing 10 μL 2 × SYBR Premix Ex Taq, 2 μL cDNA (50 ng μL^{-1}), 0.4 μL each of the 10 mM forward and reverse primers, 0.4 μL ROX and 6.8 μL PCR-grade water. Data acquisition and analysis were performed using a 7500 System SDS Software version 2.0 (Applied Biosystems). Relative gene expression levels were evaluated using $2^{-\Delta\Delta ct}$ method (Chang et al. 2009). All samples were run in triplicate, and each assay was repeated three times.