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Morphologic, physiological and immunological changes of haemocytes from *Litopenaeus vannamei* treated by lipopolysaccharide

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ABSTRACT

In this study, the cell size, viability, respiratory burst (RB) and mitochondrial membrane potential (MMP) of lipopolysaccharide (LPS)-treated haemocytes from *Litopenaeus vannamei* were measured by flow cytometry, and phenoloxidase (PO) activity of the cell supernatants was also determined. Three concentrations of LPS (1, 3 and 5 μg ml^{-1}) were used to determine the effects of LPS on haemocytes. The results revealed that haemocytes treated by LPS *in vitro* showed conspicuous dose- and time-dependent decreases in cell size and viability. The addition of trypsin inhibitor (TI) which inhibits serine proteinases further delayed the cell size reduction and cell death caused by LPS, implying that serine proteinases were involved in the cell responses. PO activities of the supernatants from LPS-treated cells were significantly higher than that from the control. These results indicated that cell size reduction caused by LPS appeared to be related to degranulation of semigranular and granular cells to release components of proPO system. RB activities were induced by LPS, but not by PMA. Sustaining and tremendous increase of RB occurred during the course of experiment, suggesting that LPS is a more efficient activator to RB of *L. vannamei* haemocytes than PMA. Specially, MMP of the LPS-treated haemocytes rose at first and then dropped, and which was significantly lower than that of control after 120 min incubation with LPS. The initial increase in MMP may have a relation to energy supply for cellular processes, and the later decrease of MMP represent cell apoptosis. Decreases on cell viability and MMP may be related to the overfull product of ROS which oxidized mitochondrial membrane and cell constituents. Though LPS stimulated PO activity of *L. vannamei*, LPS at the concentrations used in the present study is cytotoxic. These facts suggest that LPS should be carefully used as immunomodulator for shrimp.

1. Introduction

White shrimp, *Litopenaeus vannamei*, is the primary crustacean species being cultured in China. In the recent years, disease outbreaks of shrimp occurred frequently and caused severe economic loss under intensive aquaculture conditions. Aggressive pathogen is one of the reasons for these disease outbreaks (Smith et al., 2003). In order to protect the organisms from invasion of pathogen, many researchers focus on how to enhance the immunity or disease resistance in crustaceans. Some so-called 'immunomodulators' have been found and used for aquaculture, such as LPS, β-1,3-glucans and peptidoglycans (Smith et al., 2003).

Lipopolysaccharide (LPS), integral component in the outer membrane of Gram-negative bacteria, is a highly antigenic and cytotoxic substance (Schletter et al., 1995). It is also known as a powerful stimulator of innate immunity (Ulevitch and Tobias, 1995). It stimulated the proliferation of shrimp haemocytes (Sequeira et al., 1996), activated prophenoloxidase (proPO) in crayfish (Söderhäll and Häll, 1984) and shrimp (Sung et al., 1998) haemocyte lysates, induced degranulation and proPO release of haemocytes from crayfish (Cárdenas et al., 2004). Because of its known effects on the innate immune system, it also used as immunomodulator through dietary administration to improve immune functions (Takahashi et al., 2000). However, some studies reported that LPS caused a decrease in the number of circulating haemocytes which play important role in the non-special immune system of crustacean (Lorenzon et al., 1999, 2002). A *in vitro* investigation reported that LPS caused a reduction in proPO and antibacterial activities in the haemolymph of *Penaeus monodon* (Sritunyalucksana et al., 1999). Indeed, effects of this so-called 'immunostimulant' on immune and physiological responses of shrimp are also not clear, and less effort has been directed at determining its effects at a cellular level.

Because lacking of acquired immunity, crustaceans have to rely on the innate immune systems. Their innate immune systems are based largely on haemocytes (Smith, 1991). Three main haemocyte types are known in the crustacean: hyaline cells, semigranular cells and granular cells (Söderhäll and Cerenius, 1992; Van de Braak et al., 2002; Smith et al., 2003). The hyaline cells are actively phagocytic but lack of granules. By contrast the semigranular cells and granular cells mostly contain highly refractive granules. All these granules contain

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components of the proPO system, an enzyme system responsible for melanin synthesis which is known as a powerful antimicrobial component (Söderhäll and Smith, 1983; Söderhäll and Cerenius, 1998; Johansson et al., 2000; Smith et al., 2003).

The proPO system can be specifically activated by microbial cell wall components, such as LPS, β-1,3-glucan and peptidoglycan (Söderhäll and Smith, 1983; Söderhäll and Cerenius, 1998; Johansson et al., 2000; Smith et al., 2003). The pattern of activation of proPO system have been clarified in the previous studies (Söderhäll and Smith, 1983; Söderhäll and Häll, 1984; Söderhäll and Cerenius, 1998). LPS and β-1,3-glucan binding protein (LGBP) identified in crayfish (Lee et al., 2000) and shrimp (Roux et al., 2002; Cheng et al., 2005; Du et al., 2007; Lina et al., 2008) might recognize LPS as non-self molecules. After binding to their ligands, LGBP engages in the activation of the proPO activating system (Söderhäll and Smith, 1983; Söderhäll and Häll, 1984; Söderhäll and Cerenius, 1998). The basic mechanism of LPS on activation of the proPO activating system has been comparatively well characterized, but effects of LPS on cell behavior or viability have not been well considered.

Flow cytometry which analyzes the individual cell in a short time could provide an objective, reproducible and sensitive characterization of a large number of haemocytes. In order to further clarify the positive and negative effects of the so-called 'immunostimulant' LPS on crustaceans, present study applied flow cytometry to investigate the in vitro cellular responses of *L. vannamei* haemocytes to LPS. Parameters analyzed in the assessment of the cellular response to LPS were cell size, viability, RB, MMP and PO activity.

2. Materials and methods

2.1. Preparation of haemocytes

The experimental shrimps *L. vannamei* were obtained from Huangsha Live Seafood Wholesale Market (Guangzhou, Guangdong Province, P.R. China). They were maintained in the laboratory in a constant flow seawater recirculation system at 24 ± 1 °C, and acclimated at least 2 weeks prior to use. Only apparently healthy ones were used.

Haemolymph was extracted from each shrimp by a 25 ga needle and 1 ml syringe containing an equal volume of ice-cold shrimp anticoagulant buffer (450 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA-Na_2, pH 7.3) (Gollas-Galván et al., 1997). The samples were centrifuged at 800 × *g* for 10 min at 4 °C. The supernatant was discarded and the cell pellets were resuspended in ice-cold shrimp salt solution (450 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3; SSS). Then all the haemolymph samples were pooled to reduce interindividual variation and to provide enough haemocytes for LPS (from *Salmonella enterica* serotype minnesota, purified by phenol extraction, Sigma) exposure assays.

2.2. Exposure protocol

To assess the cellular response to LPS, isolated haemocytes in SSS were incubated with different LPS concentrations (1, 3 and 5 μg ml^{-1}). Control reaction mixtures contained haemocytes in SSS without LPS. To investigate the involvement of serine protease acting on the effects of LPS, trypsin inhibitor (TI, Sigma) at a final concentration of 100 μg ml^{-1} was included in a 3 μg ml^{-1} LPS reaction mixture. All the treatments were incubated at room temperature.

2.3. Flow cytometry

Flow cytometry was performed with a FACSCalibur equipped with a single argon ion laser with filtered emission at 488 nm. Photomultiplier bandpass filters for fluorescence were 530 nm (green fluorescence, FL1) and 585 nm (yellow/orange fluorescence, FL2). Size scatter height (SSC) and fluorescence data were collected on log scale, and forward scatter height (FSC) data were collected on linear scales. For each subsample, 10,000 events were counted. Cell Quest© software (Becton Dickinson Immunocytometry Systems, San Jose, CA) were used to create logical regions and colour gating analysis of fluorescence data.

2.4. Cell size and viability

Cell viability was assessed by staining cells with propidium iodide (PI, Sigma), a fluorescent DNA/RNA-specific dye which only permeates through the membranes of dead cells and stains the nucleic acids. At 20 min interval during the incubation with LPS, subsamples of 200 μl from each reaction mixtures were removed, centrifuged (800 × *g*, 10 min, 4 °C) and resuspended with 400 μl SSS (about 1×10^6 cells ml^{-1} final concentration). Then the samples were incubated at room temperature for 10 min with 10 μg ml^{-1} PI. Then both cell size and viability were analysed by flow cytometry. Results of cell size were expressed as dot plot indicating the size (forward light scatter, FSC) and the complexity (side light scatter, SSC), and distribution histogram of FSC. Cell viability was expressed as distribution histogram of PI fluorescence (FL2-H). Trypan blue exclusion assay was also done to determine the viability of LPS-treated cells.

2.5. Respiratory burst (RB)

To monitor the level of respiratory burst, the cell-permeant probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used as described by Bass et al. (1983) and Lambert et al. (2003). After it permeates through the cell membrane, DCFH-DA is deacetylated by cytosolic enzymes to dichlorofluorescin (DCFH), which is polar and trapped with the cells. Initially, DCFH was considered as a specific indicator for H_2O_2. However, later it is found to be also oxidized by nitric oxide (NO), peroxynitrite, hydroxyl (HO^-) and peroxyl (ROO^-). These reactive oxygen species (ROS) oxidizes the nonfluorescent DCFH to highly fluorescent 2′,7′-dichlorofluorescein (DCF). To directly stimulate the respiratory burst, a reaction mixture of haemocytes was incubated in 16.6 nM PMA (Sigma) (Goedken and Guise, 2004). At 30 min interval of incubation with PMA or LPS, 200 μl subsamples were centrifuged (800 × *g*, 10 min, 4 °C), resuspended with SSS, and then incubated with 5 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma) for 30 min at room temperature. Then the DCF fluorescence (FL1-H) of the haemocyte subsamples were recorded through flow cytometry. RB activity, represented as the oxidation of DCFH to DCF by ROS described above, was determined as the difference in the mean fluorescence of treated and control samples.

2.6. Mitochondrial membrane potential(MMP)

The cationic lipophilic dye, 3,3-dihexyloxacarbocyanine iodide (DIOC6, Sigma) was used to probe MMP as described by Xue et al. (2001). At 20 min interval during the incubation with LPS, 200 μl reaction mixtures were removed, centrifuged (800 × *g*, 10 min, 4 °C), resuspended with 400 μl SSS, and then incubated at room temperature for 10 min with 2 mM DIOC6. Then the haemocytes were analysed by flow cytometry, and the results were displayed as fluorescent histograms of DIOC6 (FL1-H).

2.7. Phenoloxidase (PO) activity

To render PO activity, trypsin (3 μg ml^{-1}, Sigma) was added to the haemocytes suspension. After the final time point measured in the flow cytometry experiment of cell viability, remaining cells in each of the reaction mixtures were immediately centrifuged at 800 × *g* for