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Cellular responses of the tiger shrimp *Penaeus monodon* haemocytes after lipopolysaccharide injection

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ABSTRACT

This study was aimed at investigating the *in vivo* effects of lipopolysaccharide (LPS) injection on *Penaeus monodon* haemocytes at a cellular level. Cellular responses of LPS-injected shrimp were analysed using flow cytometry. Results showed that LPS injection caused total haemocyte count (THC) and count of large cells (semigranular and granular cells) decline. In LPS-injected shrimp, percentage of large cells decreased at the initial stage, and returned to the original level later. After LPS infection, non-specific esterase activity, reactive oxygen species (ROS) production and nitric oxide (NO) production in haemocytes were significantly induced, while apoptotic cell ratio of haemocytes increased. PO activity in plasma increased in shrimp received LPS at 2 μg g^{-1} after 3–12 h and at 8 μg g^{-1} after 3–6 h, and then returned to the initial levels. These results demonstrated that LPS induced immune responses on haemocytes, including production of ROS and NO, and release of esterase and PO. On the other hand, THC reduction might be due to the ROS/NO-induced apoptosis. Haemocyte apoptosis which would eliminate damaged or weak cells and contribute to haemocyte renewal, may be a defending strategie against pathogens.

1. Introduction

Aggressive pathogens, one of the reasons for disease outbreaks, caused severe economic loss under current intensive aquaculture. Gram-negative bacteria, such as *Vibrio* species, is one of the main groups of pathogenic microorganism to shrimp. Understanding the toxicity mechanism of pathogens on shrimp has become a priority. Lipopolysaccharide (LPS), an integral component in the outer membrane of Gram-negative bacteria, which known as endotoxin, is the highly antigenic and cytotoxic substance [1]. To study the effects of LPS on shrimp would help us to further understand the pathological mechanism against Gram-negative bacteria.

In previous researches, both immune induction and toxic effects could be observed in LPS-treated crustaceans. They found that LPS induced degranulation and prophenoloxidase release of haemocytes from decapod crustaceans *in vivo* and *in vitro* [2], and also caused depletion of haemocytes, decrease of total haemocyte count (THC) and *in vitro* cell death [3–5]. Some studies showed the induction effects of LPS on gene expression levels of immune-related genes [6,7]. Our previous *in vitro* studies investigated the cellular responses of shrimp haemocytes against LPS using flow cytometry (FCM), demonstrating that LPS induced oxidative stress and apoptosis on shrimp haemocytes, suggesting that reactive oxygen species (ROS)/reactive nitrogen species (RNS)-induced Ca^{2+}-mediated apoptosis might be one of the toxicity mechanisms of LPS on shrimp haemocytes [8,9]. However, the real cellular responses of *in vivo* LPS infection have not been well considered. In order to further clarify if the effects of *in vivo* LPS infection is similar to the *in vitro* exposure, the present study applied FCM to investigate the *in vivo* cellular responses of *Penaeus monodon* haemocytes to *Escherichia coli* LPS.

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2. Materials and methods

2.1. Animals

The experimental shrimp *Penaeus monodon* (11.18 ± 1.04 g) were obtained from a commercial shrimp farm in Zhuhai, Guangdong Province, China. They were maintained in the laboratory with diluted seawater at 20‰, pH 7.9–8.0 and controlled temperature (24 ± 2 °C), with continuous water circulation. Prior to experimental use, animals were acclimated to the laboratory conditions for one week, and fed twice daily with commercial shrimp feed (40% protein, 5.0% fat, 5.0% fiber and 16% ash). Only apparently healthy shrimp in the intermoult stage were used.

2.2. LPS injection

LPS (from *Escherichia coli* 055:B5, purified by phenol extraction, Sigma) was dissolved in physiological saline solution (0.85% NaCl) to give concentrations of 0.5 and 2 μg μl^{-1}. Shrimps were randomly divided into three groups (twenty shrimp/group in separate aquaria) of different LPS doses: 0 (Shrimp injected with the same amount of sterile physiological saline solution were maintained as control), 2 and 8 μg g^{-1} wet weight. Each group was set up in triplicates. Haemolymph of each group was collected individually at 0, 3, 6, 12 and 24 h after injection.

2.3. Preparation of haemocyte suspension and total haemocyte count (THC)

Experiment was performed on individual sample. Haemolymph (400 μl) was extracted from each shrimp by a 25 gauge needle and 2.5 ml syringe containing an equal volume of ice-cold anticoagulant solution (AS, glucose 20.5 g L^{-1}, sodium citrate 8 g L^{-1}, sodium chloride 4.2 g L^{-1}, pH 7.5). A drop of diluted haemolymph sample was removed to a haemocytometer to measure THC with light microscope (Olympus). Two hundred microliter diluted haemolymph was transferred into a separate microcentrifuge tube held on ice for PO activity analysis. The remaining diluted haemolymph was diluted with AS to obtain a final concentration of about 1×10^6 cells ml^{-1}, and then used for flow cytometric analysis.

2.4. Flow cytometry

Flow cytometry was performed with a FACSCalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA) 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). Side scatter (SSC) and fluorescence data were collected on log scale, and forward scatter (FSC) data were collected on linear scales. For each haemocyte sample, 10,000 events were counted. Cell Quest® software (Becton-Dickinson, San Jose, CA, USA) was used to create logical regions and colour gating analyses of fluorescence data.

2.5. Percentage and count of semigranular and granular cells

A volume of 200 μl haemocyte suspension was directly analysed by flow cytometer as described previously [8]. FSC and SSC indicate the relative size and granularity of haemocytes respectively. Results were expressed as FSC-H/SSC-H dot plot and FSC-H distribution histogram. Haemocyte subpopulations were divided into large cells (semigranular cells and granular cells) and small cells (hyaline cells) according to the remarkable FSC boundary as described previously. Percentage of large cells was analysed, and the real number of large cells was calculated through multiply THC by the percentage.

2.6. Non-specific esterase activity

Esterase activity was measured using the non-specific liposoluble substrate fluorescein diacetate (FDA, Molecular Probes). FDA solutions were prepared by dissolving FDA in dimethyl sulphoxide (DMSO) to 0.5 mM and stored at −20 °C. A volume of 200 μL haemocyte suspension was incubated with 5 μM FDA for 30 min at room temperature (22–23 °C) in the dark. Then the fluorescence of cells was recorded on FL1 by detector of flow cytometer. Esterase activity was expressed as mean fluorescence of FDA in arbitrary units (A.U.).

2.7. Reactive oxygen species (ROS) production

To monitor the level of ROS, the cell-permeant probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) was used as described previously [9]. DCFH-DA solutions were prepared by dissolving DCFH-DA in DMSO to 1 mM and stored at −20 °C. A volume of 200 μl haemocyte suspension was incubated with 10 μM DCFH-DA for 30 min at room temperature in the dark. Then the DCF fluorescence of cells was recorded on FL1 detector of flow cytometer. ROS production was expressed as mean fluorescence of DCF in arbitrary units (A.U.).

2.8. NO production

The fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Sigma) was used to measure intracellular NO production as described previously [10]. DAF-FM DA solutions were prepared by dissolving DAF-FM DA in DMSO to 1 mM and stored at −20 °C. A volume of 200 μl haemocyte suspension was incubated with 10 μM DAF-FM DA for 60 min at room temperature in the dark. Then the DAF-FM fluorescence of cells was recorded on FL1 detector of flow cytometer. NO production was expressed as mean fluorescence of DAF-FM in arbitrary units (A.U.).

2.9. Apoptotic cell ratio

The apoptotic haemocytes were examined using Annexin V-FITC/PI apoptosis detection kit (Invitrogen) following the manufacturer's instructions [9]. Briefly, 300 μl haemocyte suspension was centrifuged and resuspended in 100 μl 1 × Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Then the haemocyte suspension was stained with 5 μl of Annexin V-FITC and 10 μl of 50 μg ml^{-1} PI for 15 min in the dark. Then 400 μl 1 × Annexin V binding buffer was added to each tube, and then the cells were analysed immediately by flow cytometry for fluorescence in the FL1 (Annexin V) and FL2 (PI) channels. Results were expressed as Annexin V-FITC/PI dot plot. Cells stained negative with both probes represent live cells (quadrant a). Cells stained positive with Annexin V-FITC and negative with PI are early apoptotic cells (quadrant b). Cells stained positive with both Annexin V-FITC and PI are in the end stage of apoptosis, undergoing necrosis, or already dead (quadrant c). Apoptotic cell ratio was expressed as the percentage of cells in quadrant b and c.

2.10. Phenoloxidase (PO) activity in plasma

PO activity was measured spectrophotometrically at 490 nm by recording the formation of dopachrome following the procedures of Huang et al. [11]. Two hundred microliter diluted haemolymph was centrifuged at 800 × g for 10 min at 4 °C, and the plasma was separated for determination. A volume of 20 μl plasma was added