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Short communication

Measurement of intracellular nitric oxide (NO) production in shrimp haemocytes by flow cytometry

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ABSTRACT

A flow cytometric method to measure the production of intracellular nitric oxide (NO) was adapted for use with shrimp haemocytes. We applied fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) for NO detection in haemocytes from the tiger shrimp *Penaeus monodon*, and used flow cytometry to quantify fluorescence intensity in individual haemocyte. The optimized protocol for intracellular NO analysis consists to incubate haemocytes with DAF-FM DA at 10 μM for 60 min to determine the mean fluorescence intensity. Result showed that NO was also produced in the untreated shrimp haemocytes. NO level in granular cells and semigranular cells were much higher than that in hyaline cells. Defined by different characteristic of NO content, three subsets of haemocytes were observed. Zymosan A at dose of 10 or 100 particles per haemocyte triggered higher DAF-FM fluorescence intensity in granular and semigranular cells, than PMA that had no significant impact on all three cell types. These results indicate that granular and semigranular cells are the primary cells for NO generation. Cytochalasin B significantly inhibited the NO level induced by zymosan A. N^G-Monomethyl-L-arginine (L-NMMA) and diphenylene iodonium chloride (DPI) significantly suppressed the DAF-FM fluorescence in haemocytes, but apocynin could not modulate it, indicating that the DAF-FM fluorescence was closely related to the activity of NO-synthase pathway. The NO donor sodium nitroprusside (SNP) improved the DAF-FM fluorescence in haemocytes, while the NO scavenger C-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) significantly decreased the fluorescence, demonstrating that the fluorescence intensity of DAF-FM is mainly dependent on the intracellular NO level.

1. Introduction

Nitric oxide (NO) has been identified as a vital physiological modulator and a signaling molecule in mammals [1]. In marine invertebrates, biological roles of NO were found to be related to feeding, immune defence, environmental stress, learning, metamorphosis, swimming, symbiosis, haemocyte aggregation and regulation of blood pressure [2]. Recent studies found that NO acts as a cytotoxic molecule contributed to microorganisms killing in crustaceans [3–6], as in mammalians [1]. However, the information about biological role of NO in crustacean is still scarce.

The direct detection of NO content in biological samples is a challenge due to its short half-life and low concentration. Several indirect methods have been applied to detect the NO level. The most prevalent method of NO production assay was the Griess colorimetric reaction, a method that quantifies nitrites (NO_2^-) and nitrates (NO_3^-), the stable products resulting from the degradation of NO [3,4]. However, there are at least two major restriction of this method: 1) This indirect method may not reflect the real NO level. Not all the NO_2^-/NO_3^- in aquatic animals come from NO metabolism. Environmental NO_2^- tends to accumulate into the blood through active uptake mechanisms associated with the chloride cells of the gills [7,8]. 2) This indirect method could not detect the instantaneous NO content in individual cell, and it is hard to estimate the major cell source of NO.

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Another common method for NO detection was flow cytometric assay using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) [9,10]. This probe could also be oxidized by reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), peroxynitrite, hydroxyl (HO^-) and peroxyl (ROO^-) [9]. Specific inhibitors should be included in this indirect method. Thus, result may also be affected by some processes, such as the dose of inhibitor. Moreover with, this qualitative measure is hard to quantify the NO content accurately. The currently used methods are neither sensitive nor specific. A direct method which is more simple, sensitive and accurate is needed.

Flow cytometry (FCM) has been proposed for direct detection of intracellular NO production in human [11], mammal [12] and mussel [13] cells using NO-specific fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) or more sensitive probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). This direct detection could overcome the major restriction of the indirect methods. Furthermore, FCM can provide information at the single-cell level, so it can easily be applied to analyse the production of NO in different cell subpopulations.

The aim of the present work was to describe the development of a flow cytometric approach to measure the NO production in shrimp haemocytes using the fluorescent probe DAF-FM DA, and to evaluate the effects of various modulators on the NO production by different haemocyte types.

2. Materials and methods

2.1. Animals

The experimental shrimp *Penaeus monodon* (7.78 ± 0.82 g) were obtained from a commercial shrimp farm in Nansha, Guangzhou, 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. Only apparently healthy shrimp in the intermoult stage were used.

2.2. Preparation of haemocyte suspension

Haemolymph (300 μl) was extracted from each shrimp with 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). The diluted haemolymph from each shrimp was transferred into a separate microcentrifuge tube held on ice.

2.3. Measurement of intracellular nitric oxide by flow cytometry

FCM was performed with a FACSCalibur instrument (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). Size scatter height (SSC) and FL1 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.

4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Sigma) is a NO-specific fluorescent probe, which is cell-permeant and passively diffuses across cellular membranes. Once inside cells, it is deacetylated by intracellular esterases to become DAF-FM. After specifically reacting with NO, DAF-FM is further converted to DAF-FM triazole (λ excitation = 495, λ emission = 515) which exhibits about a 160-fold greater fluorescence quantum efficiency [12]. DAF-FM green fluorescence was measured on FL1 detector of flow cytometer. Results were given as mean of fluorescence in arbitrary units (A. U.).

2.4. DAF-FM DA dose and time responses

An initial experiment was performed to define the optimum DAF-FM DA dose to be added to haemocyte suspension; this dose should be sufficient to emit a measurable fluorescent signal on the FL1 detector and to be non-toxic for cells.

After haemolymph was extracted as described previously, haemolymph samples were pooled to reduce interindividual variation, and then the cell concentration was adjusted to about 1×10^6 cells ml^{-1} with AS. The diluted pooled haemolymph sample was divided into four sub-samples of 2 ml, then 20 μl of the DAF-FM DA stock solutions (0.1, 0.5, 1 and 5 mM) was added to yield final concentrations of 1, 5, 10 and 50 μM, respectively. The mixture was incubated in the dark at room temperature. DAF-FM fluorescence of haemocyte was evaluated just after DAF-FM DA addition ($t = 0$) and at $t = 15$, 30, 45, 60, 75 and 90 min. Cell viability at the end point of the experiment was determined using propidium iodide (PI, Sigma) stain as described previously [14]. This experiment repeated three times.

2.5. Non-induced nitric oxide production in different haemocyte types

Haemolymph was extracted as described previously, and then was diluted with AS to obtain a final concentration of about 1×10^6 cells ml^{-1}. This experiment was performed on individual samples, and fifteen shrimp were analysed. Two hundred microlitres of haemocyte suspension from each shrimp was incubated with DAF-FM DA at 10 μM (the optimum dose) for 60 min (the optimum time) in the dark. Three different morphologic haemocyte types (hyaline cells, semigranular cells and granular cells) can be defined basing on the relative size (FSC values) and granularity (SSC values) as described previously [14]. The DAF-FM fluorescence intensity of each haemocyte type was analysed.

2.6. Haemocyte responses to modulators

Effects of two activators, four inhibitors, NO donor and scavenger on DAF-FM fluorescence intensity were determined in the tiger shrimp haemocytes. Species, doses and functions of modulators used in this study are presented in Table 1.

2.6.1. Haemocyte responses to activators

Two possible NO activating agents were tested: zymosan A (Sigma) and phorbol myristate acetate (PMA, Sigma). Zymosan A stock preparation: 200 mg of zymosan A was suspended in 10 ml of AS, heated in a boiling water bath for 30 min, and then washed twice. The stock suspension was resuspended in a range of volumes of AS to make three suspensions that contained increasingly higher concentrations of zymosan particles. The particle count was checked microscopically, and aliquots were frozen at −20 °C.

After haemolymph was extracted, pooled and diluted as described previously, zymosan A was added to haemocyte suspensions to obtain approximately 1, 10 and 100 particles per haemocyte. PMA was added to obtain 1 and 10 μg ml^{-1}. DAF-FM DA was added to haemocyte suspensions to yield final concentration of 10 μM, 30 min after adding zymosan A or PMA. The haemocyte solutions were incubated in the dark for 60 min at room temperature, and then the DAF-FM fluorescence of each haemocyte type was analysed by flow cytometry.