Studies on nitric oxide synthase activity in haemocytes of shrimps *Fenneropenaeus chinensis* and *Marsupenaeus japonicus* after white spot syndrome virus infection

Guojian Jiang [a], Rencheng Yu [b,*], Mingjiang Zhou [b]

[a] *Marine Development Academy, Ocean University of China, Qingdao 266003, PR China*
[b] *Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, PR China*

Abstract

The nitric oxide synthase (NOS) activity in the haemocytes of shrimps *Fenneropenaeus chinensis* (Osbeck) and *Marsupenaeus japonicus* (Bate) was studied after white spot syndrome virus (WSSV) infection to determine its characteristics in response to virus infection. First, the NOS activity in haemocytes of shrimps was determined by the means of NBT reduction and changes in cell conformation. And the variations of NOS activity in shrimps after challenge with WSSV intramuscularly were evaluated through the analysis of L-citrulline and total nitrite/nitrate (both as NO derivates) concentrations. The result showed that NOS activity in the haemocytes of *F. chinensis* increased slightly from 0 to 12 h postchallenge, indicated by the variations of L-citrulline (from 11.15 ± 0.10 to $12.08 \pm 0.64\,\mu M$) and total nitrite/nitrate concentrations (from 10.45 ± 0.65 to $12.67 \pm 0.52\,\mu M$). Then it decreased sharply till the end of the experiment (84 h postchallenge), the concentrations of L-citrulline and total nitrite/nitrate at 84 h were 1.58 ± 0.24 and $2.69 \pm 0.70\,\mu M$, respectively. The LPS-stimulated NOS activity kept constant during the experiment. However, in *M. japonicus*, the NOS activity kept increasing during the first 72 h postchallenge, the concentrations of L-citrulline and total nitrite/nitrate increased from 7.82 ± 0.77 at 0 h to $10.79 \pm 0.50\,\mu M$ at 72 h, and from 8.98 ± 0.43 at 0 h to $11.20 \pm 0.37\,\mu M$ at 72 h, respectively. Then it decreased till the end of the experiment (216 h postchallenge), and the concentrations of L-citrulline and total nitrite/nitrate at 216 h were 5.66 ± 0.27 and $4.68 \pm 0.16\,\mu M$, respectively. More importantly, an apparent increase of LPS-stimulated NOS activity was observed in *M. japonicus* at 48 h postchallenge, which was about 4 times higher than that in the control group of health shrimps. In correspondence with the difference of NOS activity between the two species of shrimps, the cumulative mortalities of the shrimps were also different. All shrimps of *F. chinensis* in the mortality experiment died in 66 h, much more quickly than *M. japonicus*, whose accumulative mortality reached 100% after 240 h. Data here reported let us hypothesize that NOS activity in the haemocytes of shrimps *F. chinensis* and *M. japonicus* responses to WSSV infection differently, and this might be one of the reasons for the different susceptibility of *F. chinensis* and *M. japonicus* to WSSV infection.
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Keywords: *Fenneropenaeus chinensis* (Osbeck); *Marsupenaeus japonicus* (Bate); White spot syndrome virus; Nitric oxide synthase

Penaeid shrimp culture is a worldwide economic activity. However, the intensification and environment degradation of shrimp farming have been accompanied by the development of many infectious diseases, especially from viral origin, which cause a dramatic decrease in shrimp production [1,2]. White spot syndrome virus (WSSV), a circular double-strand DNA virus [3–6], has led to heavy crop loss and continues to seriously affect the cultured penaeids in the shrimp farming systems around the world [7]. In this context, control of disease is very important to insure the long-term survival of shrimp aquaculture. Therefore, it is necessary to study the immune factors of shrimp in response to the invasion of WSSV, which may be helpful in understanding the mechanism of shrimp immunity to WSSV infection and developing strategies for management of the disease.

Belonging to invertebrate, shrimp lack a true adaptive immune response system [8]. However, living in an aquatic environment rich in microorganisms, shrimps have developed effective systems for detecting and eliminating noxious microorganism depending on non-specific innate immune response based on the activity of haemocytes, including phagocytosis, phenoloxidase releasing, bactericidin synthesis, and so on [2]. Non-specific molecules like reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) are also involved. Surprisingly, in shrimps, the function and immune status of nitric oxide synthase (NOS) and its catalyzing products—nitric oxide, have been seldom reported. Nitric oxide is a very important component of innate immune system, which has the antiviral, anti-bacterial, and anti-parasite properties by damaging the DNA, enzyme and membrane of pathogen directly or indirectly through the action on the bases and strands of DNA, protein and lipid of membrane [9–12]. Therefore, the role of NO and NOS in disease resistance of shrimps is worth studying. In this paper, we have tried different methods to determine NOS activity in the haemocytes of *Fenneropenaeus chinensis* and *Marsupenaeus japonicus* qualitatively and quantitatively, and the role of the NOS in haemocytes of shrimps is also discussed in response to virus infection.

Experimental procedures

Animals

Marsupenaeus japonicus and *F. chinensis* were obtained from Ocean Shrimp Farm in Qingdao, China, with average length of 14.43 ± 0.86 cm (mean $\pm$ SD, $N = 80$) and 16.70 ± 1.40 cm (mean $\pm$ SD, $N = 80$), and average weight of 19.84 ± 3.71 g (mean $\pm$ SD, $N = 80$) and 25.90 ± 7.05 g (mean $\pm$ SD, $N = 80$), respectively. They were fed with artificial diet twice a day and acclimated in aerated flat-bed tanks containing sand filtrated sea water (20 ± 1 °C; salinity, 30‰) for 7 days prior to experiment. Every day the unconsumed food and faeces were removed carefully with a siphon. All shrimps used in experiment were intermoult males.

Chemicals

All chemicals used in the experiment were A.R (Analytical reagent) grade or better. The glassware was pyrogen-free to avoid the enzymatic interruption by endotoxin.

Haemocytes sample preparation

Haemolymph of shrimp was collected from the ventral-sinus with a 26-gauge needle and 1-ml syringe containing pre-cooled (4 °C) anti-coagulant solution [13] (450 mM NaCl, 10 mM KCl, 10 mM EDTA-Na$_2$, and 10 mM HEPES, pH 7.3, 850 mOsmol/kg, sterilized by filtration (0.2 μm) before using). The mixture was then injected into a 50 ml centrifuge tube containing anti-coagulant solution. The solution was mixed thoroughly and centrifuged at 600g for 10 min at 4 °C. The supernatant was removed and haemocytes were adjusted to a certain cell density and resuspended in L-arginine solution (0.1 M Bis–Tris buffer containing 5 mM L-arginine (Sigma) and 5 mM Ca^{2+}) [14] to make haemocytes suspension for NOS activity analysis.

Identification of NOS activity in haemocytes of shrimps

NBT reduction method

The NOS activity in haemocytes of shrimps was determined with NBT reduction method, according to Weiske and Wiesner [15]. The procedure was introduced briefly as below: 500 μl LPS solution [0.1 M Bis–Tris buffer containing 200 μg/ml LPS (Sigma L2630)] [14] was mixed with 500 μl haemocytes suspension with cell density of 10^6 and incubated at 4 °C for 4 hours. The mixture was then centrifuged at 600g for 2 min at 4 °C, and the supernatant was discarded. The residue haemocytes were fixed with 4% formaldehyde (dissolved in 0.1 M Tris buffer with final pH of 7.5–8.0) for 20 min, and the cells were washed at least twice with 0.1 M Tris buffer [pH 7.8, containing 0.1% Triton X-100 (Serva 37240)]. The cells were then resuspended in the freshly prepared 0.01% NBT (Sigma N6876) solution [in 0.1 M Tris buffer containing 0.1% Triton X-100 and 0.01% NADPH (Sigma N1630)] and incubated for 10 min at room temperature. Subsequently the mixture was centrifuged at 600g for 5 min at 4 °C, and the supernatant was replaced with 0.7 ml of 70% methanol. After intensive mixing and a further centrifugation step at 600g for 10 min at room temperature, the methanol was replaced with 0.6 ml DMSO solution [54% (v/v) dimethyl sulfoxide (Sigma D8779) and 46% (v/v) 2 N KOH] to dissolve the formazan. After a final incubation for 30 min at room temperature with intensive mixing at 10 min intervals, the contents were centrifuged at 10,000g for 5 min at room temperature, and the absorbance of the resulting supernatants was determined at 670 nm (BECKMAN DU 650 spectrophotometer). The NADPH in the 0.01% NBT solution was replaced with NADH (Sigma N8129) was used as control.

Haemocyte conformation approach

The NOS activity in haemocytes of shrimps was also determined by haemocyte conformation approach, according to Ottaviani et al. [16]. Briefly, 25 μl mixture of anti-coagulant and haemolymph with cell density of 10^6 was placed on a dichromate cleaned glass slides and incubated in humidified chamber for 30 min at room temperature. The slides were then rinsed with 0.1 M Bis–Tris buffer to remove the anti-coagulant and the cells that did not adhere to the slide. Subsequently the slides were divided into four groups (A–D). Group A was added with 25 μl *Escherichia coli* suspension at density of 1×10^8 cells/ml, group B was added with 25 μl *E. coli* suspension and 25 μl LPS solution, group C was added with 25 μl *E. coli* suspension, 25 μl LPS solution, and 25 μl L-NMMA (N^ω-monomethyl-L-arginine,

Sigma M7033) solution with final concentration of 800 μM, and group D was added with 25 μl *E. coli* suspension and 25 μl SNP (sodium nitroprusside, Sigma) solution with final concentration of 10 mM. After incubation in humidified chamber for 4 h, samples were stained with toludine blue solution (0.5% toludine blue in 3 mM borate solution) for 5 min, and rinsed with water for 1 min. The samples were then observed under microscope (Zeiss, Jena, Germany) at a magnification of 400.

Analysis of NOS activity in haemocytes of shrimps

NOS activity was analyzed quantitatively by determination of L-citrulline and total nitrite/nitrate concentrations. Briefly, 500 μl haemocytes suspension with cell density of 10^5 cell/ml was mixed with 500 μl LPS solution, and incubated at 4 °C for 8 h [14]. Then, the haemocytes were lysed by adding 1% Triton X-100 and agitating on a vortex mixer. After centrifugation at 13,000*g* for 15 min at room temperature, the supernatants were removed and ultrafiltrated. The filtrate was used to determine the concentration of L-citrulline and total nitrite/nitrate. The haemocytes not incubated with LPS were used as control. The differences in NOS activity between the LPS treated haemocytes and the control was considered as LPS stimulated NOS activity.

Determination of L-citrulline

L-Citrulline was determined according to Marzinzig et al. (1997) [17] with modifications described in Weiske and Wiesner (1999) [15]. For each sample, 150 μl filtrate was mixed with 50 μl urease (Sigma U2125) solution (165 U/ml) and incubated at 37 °C for 30 min. Then, 50 μl of 2.45 M trichloroacetic acid was added and mixed thoroughly. After centrifugation at 13,000*g* for 15 min at room temperature, 150 μl supernatant was collected and mixed with 450 μl ADMS reagent [18% (v/v) 47.7 mM anti-pyrine (Sigma A5882) in distilled water + 40% (v/v) 79 mM diacetyl monoxime (Sigma B0753) in 83 mM acetic acid + 42% (v/v) 15 N H_2SO_4]. The mixture was incubated for 25 min in a boiling water bath. Samples were cooled to room temperature in the dark and then centrifuged at 13,000*g* for 5 min, and the absorbance of the supernatant was measured at 450 nm (BECKMAN DU 650 spectrophotometer). The concentration of L-citrulline (Sigma C7629) was calculated according to the standard curve of L-citrulline prepared.

Determination of total nitrite/nitrate

Nitrite/nitrate determination was carried out according to Marzinzig et al. [17] with improvements described in Weiske and Wiesner [15]. For each assay, 300 μl of ultrafiltrated supernatant was mixed with 20 μl Bis–Tris buffer containing FAD [flavin adenine dinucleotide (Sigma F6625), final concentration 5.5 μM], NADPH (final concentration 30.0 μM), and nitrate reductase (Sigma N7265, final concentration 0.1 U/ml). The mixture was incubated for 60 min at 37 °C under dark condition [18]. Then, 20 μl Bis–Tris buffer containing L-lactic dehydrogenase (Sigma L2500, final concentration 0.1 kU/ml) and sodium pyruvate

(Sigma P2256, final concentration 0.3 mM) were added and incubated for another 30 min at 37 °C. After centrifugation at 10,000*g* for 15 min at 4 °C, 300 μl supernatant was mixed thoroughly with 150 μl dapsone solution (Sigma D2505, final concentration 4.7 μM), and 150 μl NEDA [*N*-(1-naphthyl)ethylenediamine, Sigma N9125] solution (final concentration 1 mM) was added 5 min later. The resulted solution was centrifuged at 10,000*g* for 5 min at 4 °C, and the absorbance of supernatant was measured at 550 nm (BECKMAN DU 650 spectrophotometer). The concentration of total nitrite/nitrate was calculated according to the standard curve of nitrite prepared.

Comparative study of NOS activity in F. chinensis and M. japonicus after WSSV-infection

Preparation of WSSV stock solution

The carapace, appendages, and hepatopancreas were discarded from frozen *F. chinensis* with severe WSSV-infections. The residual tissues were then ground into pieces and 1 g tissue was taken to mix with 4 ml of PPB [19] (NaCl, 23.0 g/L; K_2SO_4, 1.1 g/L; $CaCl_2$, 1.6 g/L; $MgSO_4 \cdot 7H_2O$, 1.6 g/L; $NaH_2PO_4 \cdot 2H_2O$, 0.35 g/L; and $NaHCO_3$, 0.05 g/L; pH 6.5; osmolarity, 867.9 mOsmol/L, sterilized by filtration (0.2 μm) before using). The mixture was homogenized at 3000 rpm for 5 min and then centrifuged at 5520*g* for 30 min at 4 °C. The supernatant was centrifuged at 9820*g* for 30 min at 4 °C for 2 times. Then, the supernatant was centrifuged at 15,300*g* for 60 min at 4 °C, the precipitate diluted by PPB was used as a virus stock solution.

Experimental treatments

After acclimation, 80 shrimps were injected intramuscularly with 0.1 ml diluted virus stock solution (100×) in the lateral area of the fourth abdominal segment. The infected shrimps were then subdivided randomly into two groups and placed in two aquaria (200 L) with flow-through seawater supply. One group of shrimps was used for determination of NOS activity, and the other group was used for mortality observation. Another 80 shrimps injected with 0.1 ml PPB solution were used as control. The shrimps were fed with artificial diet twice a day, the unconsumed food and faeces were removed carefully with a siphon once a day.

Haemolymph sample collection and analysis of NOS activity

For both infected and health shrimps, haemolymph sample was collected from three shrimps each time and mixed together, as described in the paragraph of *Haemocytes sample preparation*. The haemocytes were adjusted to a cell density of 10^5 cells/ml and resuspended in L-arginine solution. The mixture was divided into six Eppendorf micro-centrifuge tubes, three of them were incubated with LPS, the other three were used as control, as described in the paragraph of Analysis of NOS activity in haemocytes of shrimps. For each tube, both L-citrulline and total nitrite/nitrate were analyzed.

The NOS activity of *M. japonicus* were assayed at 0, 24, 48, 72, 96, 120, 144, 168, 192, and 216 h postchallenge, and that of *F. chinensis* were assayed at 0, 12, 36, 60, and 84 h postchallenge, depending on the surviving time of the shrimps in the experiment.

Diagnosis of WSSV in M. japonicus and F. chinensis

Diagnosis of WSSV was performed by Digoxigenin Labeled DNA Probe Dot-blot hybridization Kit purchased from the Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. Following blood sampling for analysis of NOS activity, gills of the same shrimps collected were used for WSSV detection [20].

Statistics

Data are reported as means ± SE for each treatment, respectively. The significance of variations between different treatments or groups was analyzed by Student's *t* test or ANOVA in software Excel. The variation was considered to be significant if $P < 0.01$.

Results

Identification and analysis of NOS activity in haemocytes of shrimps

Both NBT method and haemocyte conformation approach confirmed the NOS activity in haemocytes of the shrimps. The results of NBT method showed that LPS stimulated the activity of NOS in both species of shrimps, after fixation with 4% formaldehyde, with the absorbance value significantly higher than the control ($P < 0.001$). For *F. chinensis* and *M. japonicus*, the absorbance value increased from $0.091 ± 0.006$ to $0.160 ± 0.008$ and from $0.106 ± 0.021$ to $0.458 ± 0.048$, respectively.

The results of haemocyte conformation approach also confirmed the NOS activity in the haemocytes of both species. Most of the haemocytes without any treatment were in amoeboid shape with sporadic bacteria congregated or clumped in their vicinity, as shown in Figs. 1A and 2A. However, most of the haemocytes incubated with LPS became round instead of amoeboid, with a lot of bacteria aggregated around the haemocytes (Figs. 1B and Fig. 2B). The phenomenon was the same as those haemocytes treated with SNP, a donor of NO, which suggested the production of NO stimulated by addition of LPS (Figs. 1D and Fig. 2D). This process could be effectively blocked by the addition of NOS inhibitor L-NMMA, which showed similar results with the haemocytes not treated with LPS (Fig. 1C, Fig. 2C). These results suggested the existence of NOS in haemocytes of shrimps, and the enzyme activity could be stimulated by the addition of LPS, and blocked by L-NMMA.

The two methods for the analysis of NOS activity are based on the principle that NOS can catalyze L-arginine to generate L-citrulline and NO quantitatively, therefore, concentrations of L-citrulline or total nitrite/nitrate
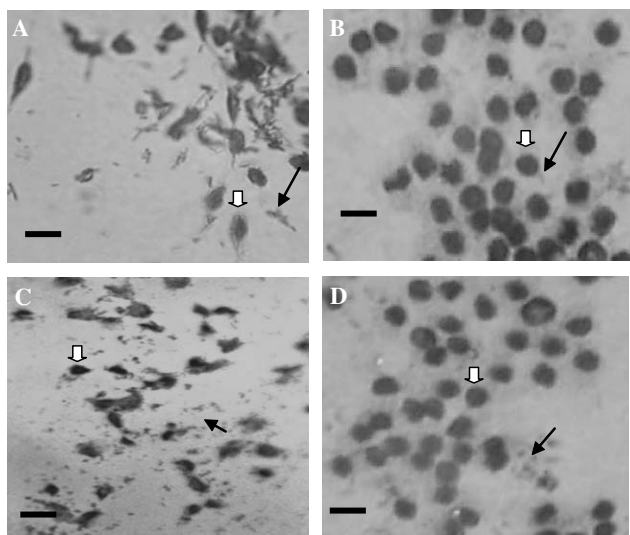

Fig. 1. Haemocytes of *F. chinensis* under different treatments (scale bar: 10 μm, (⊡) haemocyte, (→) *E. coli*.) (A) 4 h post-incubation with *E. coli*. (B) 4 h post-incubation with LPS and *E. coli*. (C) 4 h post-incubation with L-NMMA, LPS and *E. coli*. (D) 4 h post-incubation with SNP and *E. coli*.

Fig. 2. Haemocytes of *M. japonicus* under different treatments (scale bar: 10 μm, (⊡) haemocyte, (→) *E. coli*.) (A) 4 h post-incubation with *E. coli*. (B) 4 h post-incubation with LPS and *E. coli*. (C) 4 h post-incubation with L-NMMA, LPS, and *E. coli*. (D) 4 h post-incubation with SNP and *E. coli*.
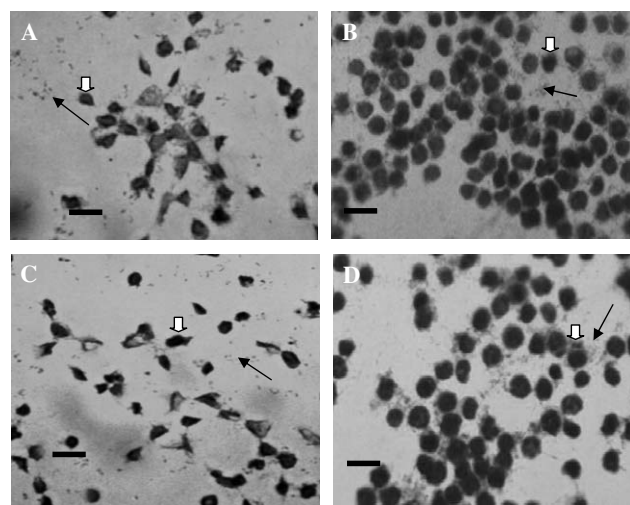
(product of NO) determined could be used to evaluate the activity of NOS. Our results suggested that the two methods coincide well with each other, and either of the methods could be used for the analysis of NOS activity in haemocytes of shrimps.

Variations of NOS activity in F. chinensis and M. japonicus after WSSV-infection

In the group of *F. chinensis* infected by WSSV, the results showed that NOS activity in haemocytes increased slightly from 0 to 12 h postchallenge, indicated by the variations of L-citrulline (from $11.15 ± 0.10$ to $12.08 ± 0.64$ μM) and total nitrite/nitrate concentrations (from $10.45 ± 0.65$

to $12.67 \pm 0.52\,\mu M$). Then, the NOS activity decreased sharply till the end of the experiment, and the concentrations of L-citrulline and total nitrite/nitrate at 84 h were 1.58 ± 0.24 and $2.69 \pm 0.70\,\mu M$, respectively (Fig. 3). The LPS-stimulated NOS activity kept constant during the experiment (Table 1). In contrast, both the NOS activity and LPS-stimulated NOS activity in the health shrimps used as control was relatively stable during the experiment, and the LPS-stimulated NOS activity in healthy shrimps is generally higher than that in the WSSV-infected shrimps (Fig. 3 and Table 1).

In the group of *M. japonicus* infected by WSSV, the NOS activity in haemocytes kept increasing during the first 72 h postchallenge, the concentrations of L-citrulline and total nitrite/nitrate increased from 7.82 ± 0.77 at 0 h to $10.79 \pm 0.50\,\mu M$ at 72 h, and from 8.98 ± 0.43 at 0 h to $11.20 \pm 0.37\,\mu M$ at 72 h, respectively. The NOS activity in the haemocytes of infected shrimps kept higher than the control from 0 to 120 h postchallenge. Then it decreased till the end of the experiment, and the concentrations of L-citrulline and total nitrite/nitrate at 216 h were 5.66 ± 0.27 and $4.68 \pm 0.16\,\mu M$, respectively (Fig. 4). For LPS-stimulated NOS activity, an apparent increase was observed during the first 48 h, and the highest value at 48 h postchallenge was about 4 times higher than that in the control group of health shrimps (Table 2). The LPS-stimulated NOS activity after 48 h then decreased dramatically and became lower than the control. For the health shrimps used as control, the NOS activity and LPS-stimulated NOS activity were relatively stable during the experiment (Fig. 4, Table 2). Compared to *F. chinensis*, the NOS activity in the haemocytes of *M. japonicus* was more potential to be stimulated by LPS at the early stage of infection ($P < 0.01$). The value of LPS-stimulated NOS activity was much higher than that of *F. chinensis* ($P < 0.01$, Figs. 3 and 4).

Diagnosis of WSSV

The results of Dot-blot hybridization test showed that both species of shrimp were free of WSSV infection prior to experiment, and the shrimps used as control in the experiment were also free of WSSV infection. However, the infection of WSSV in both species after WSSV injection could be confirmed by the method. The virus was detected at 72 h in *M. japonicus* and at 36 h in *F. chinensis* after virus injection, which was in correspondence with the difference of NOS activities in the two species.

Cumulative mortalities of WSSV infected M. japonicus and F. chinensis

In correspondence with the difference of NOS activity between the two species, the cumulative mortalities were also different. All shrimps of *F. chinensis* in the mortality experiment died in 66 h, much more quickly than *M. japonicus*, whose accumulative mortality reached 100%
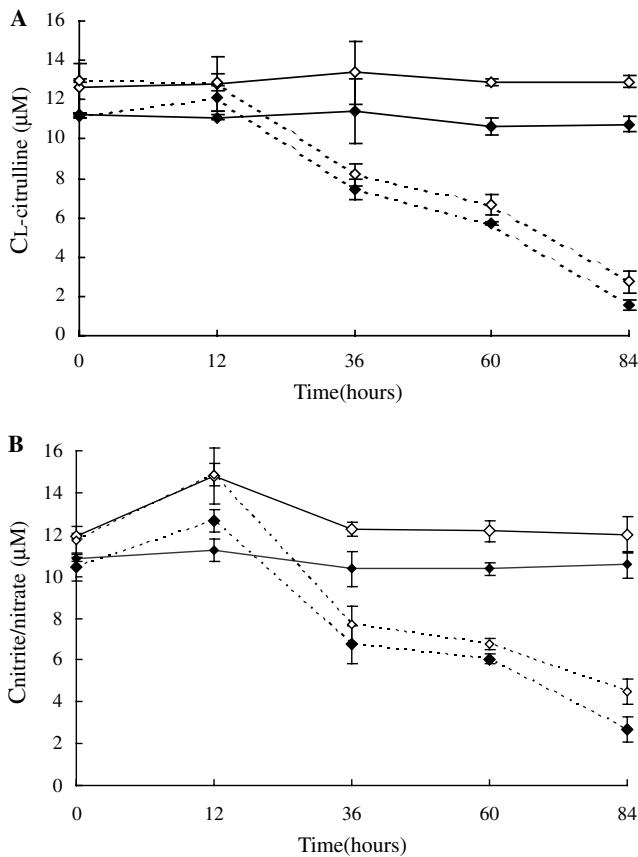

Fig. 3. Variation of NOS activity in haemocytes of *F. chinensis* after WSSV infection. ((—◆—) NOS activity in haemocytes of healthy shrimps after incubation with L-arginine for 8 h, (—◇—) NOS activity in haemocytes of healthy shrimps after incubation with L-arginine and LPS for 8 h, (- -◇- -) NOS activity in haemocytes of WSSV infected shrimps after incubation with L-arginine for 8 h, (- -◆- -) NOS activity in haemocytes of WSSV infected shrimps after incubation with L-arginine and LPS for 8 h.) (A) Variation of NOS activity indicated by the analytical results of L-citrulline. (B) Variation of NOS activity indicated by the analytical results of total nitrite/nitrate.

Table 1
Comparison of LPS stimulated NOS activity (indicated by L-citrulline and total nitrite/nitrate concentrations) between the healthy and WSSV-infected shrimps of *F. chinensis*

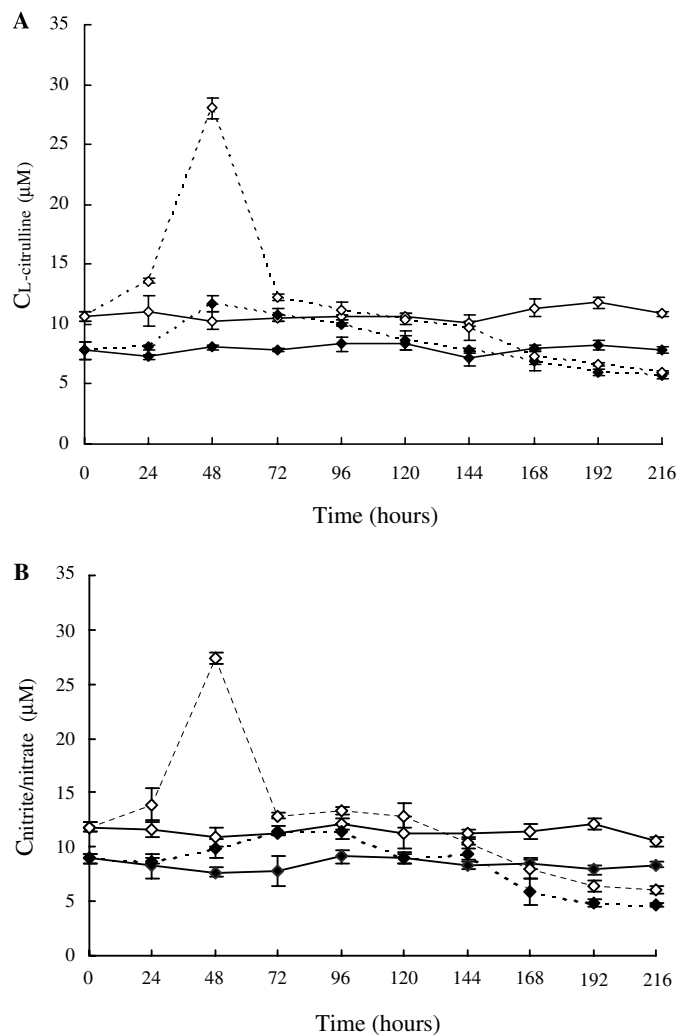|		Time (h)				
		0	12	36	60	84
Concentration (μM)						
L-citrulline	Healthy shrimps	1.37 ± 1.16	1.70 ± 1.29	1.96 ± 0.01	2.19 ± 0.26	2.14 ± 0.12
	Infected shrimps	1.81 ± 0.01	0.80 ± 0.20	0.75 ± 0.01	0.96 ± 0.41	1.16 ± 0.35
Total	Healthy shrimps	1.09 ± 0.01	3.54 ± 0.80	1.88 ± 0.50	1.82 ± 0.19	1.40 ± 0.20
Nitrite/nitrate	Infected shrimps	1.27 ± 0.12	2.12 ± 0.13	0.93 ± 0.01	0.72 ± 0.04	1.78 ± 0.08

Fig. 4. Variation of NOS activity in haemocytes of *M. japonicus* after WSSV infection. ((—◆—) NOS activity in haemocytes of healthy shrimps after incubation with ʟ-arginine for 8 h, (—◇—) NOS activity in haemocytes of healthy shrimps after incubation with ʟ-arginine and LPS for 8 h, (--◇--) NOS activity in haemocytes of WSSV infected shrimps after incubation with ʟ-arginine for 8 h, (--◆--) NOS activity in haemocytes of WSSV infected shrimps after incubation with ʟ-arginine and LPS for 8 h.) (A) Variation of NOS activity indicated by the analytical results of ʟ-citrulline. (B) Variation of NOS activity indicated by the analytical results of total nitrite/nitrate.

after 240 h. These results of mortality suggested that *F. chinensis* was more sensitive to WSSV infection than *M. japonicus*.

Discussion

In the present study, we examined the NOS activity in the haemocytes of shrimps after LPS stimulation, and the variation of NOS activity after WSSV infection were also studied and compared between two species of shrimps, *F. chinensis* and *M. japonicus*.

NOS has been extensively studied among vertebrates and invertebrates. Some of the recent researches have confirmed the presence of NOS activity in haemocytes of invertebrates [15,17,21,22,29,30]. Among these researches, techniques of analytical chemistry, immunohistochemistry, and cell and molecular biology have been used to identify NOS [15,17,23–28]. In this experiment, the NBT reduction and cellular conformation approach were employed to identify NOS in the haemocytes of *M. japonicus* and *F. chinensis*, under the stimulation of LPS. Both of the methods could confirm the presence of NOS activity in the two species of shrimps, as reported previously in haemocytes of other invertebrates, like *Mytilius edulis* and *Viviparus ater* [15,22]. However, the expression of NOS in haemocytes still needs to be further confirmed with techniques like immunochemistry or polymerase chain reaction (PCR).

In vertebrates, three isoforms of NOS, which can be divided into two functional classes, have been found so far. The Ca^{2+}/calmodulin-dependent neuronal NOS (nNOS) and endothelial NOS (eNOS) belong to the constitutive class. They produce NO in short bursts in low concentrations for physiological purposes. The other class, which is mainly composed of Ca^{2+}-independent inducible NOS (iNOS), is related to the diseases resistance of the organisms. iNOS found in innate immune cells and non-immune cells, such as macrophages and epithelial cells, can produce NO in high concentrations for as long as the enzyme is activated. The NO produced has anti-microbial actions against various intruding microbes, mediated by oxygen radicals, and active oxygen species through the formation of reactive nitrogen oxides like peroxynitrite ($ONOO^-$), via a variety of mechanisms. However, in invertebrates, only Ca^{2+}/calmodulin dependent form was found so far [28,31–37]. And in crustaceans, NOS was reported mainly in the nervous system, regulating neuronal activity [38–49]. Recently, Kim [28] reported that NOS could also be expressed in the Y-organ, gill, testis and ovary of the land crab, which suggested that NO was also involved in other physiological processes except for neuromodulation.

Table 2
Comparison of LPS stimulated NOS activity (indicated by ʟ-citrulline and total nitrite/nitrate concentrations) between the healthy and WSSV-infected shrimps of *M. japonicus*

		Time (h)									
		0	24	48	72	96	120	144	168	192	216
Concentration (µM)											
ʟ-citrulline	Healthy shrimps	2.83 ± 0.31	3.81 ± 1.08	2.23 ± 0.56	2.69 ± 0.11	2.21 ± 0.41	2.28 ± 0.36	2.90 ± 0.42	3.28 ± 0.48	3.50 ± 0.01	3.00 ± 0.06
	Infected shrimps	2.83 ± 0.31	5.58 ± 0.06	16.31 ± 0.26	1.43 ± 0.25	1.18 ± 0.61	1.72 ± 0.30	1.90 ± 0.87	0.40 ± 0.13	0.70 ± 0.11	0.36 ± 0.13
Total	Healthy shrimps	2.86 ± 0.04	3.27 ± 0.44	3.27 ± 0.43	3.39 ± 1.24	3.03 ± 0.07	2.40 ± 1.07	2.88 ± 0.01	3.03 ± 0.10	4.20 ± 0.08	2.16 ± 0.09
Nitrite/nitrate	Infected shrimps	2.86 ± 0.04	5.21 ± 1.26	17.45 ± 0.44	1.39 ± 0.06	1.82 ± 0.34	3.80 ± 0.63	1.10 ± 0.25	2.08 ± 0.39	1.52 ± 0.14	1.40 ± 0.25

It was also suggested by Kim that the localization of NOS in connective tissue and epithelia in Y-organ and gill might function in an immune response to pathogens. The expression of NOS caused by bacterial infection in haemocytes of insects, suggesting the potential role of NOS related to disease resistance, have also been reported previously [15,50]. Here in our experiment, only a slight increase in NOS activity could be observed in the two species of shrimps shortly after WSSV infection, and then it decreased till the end of the experiment, probably due to the destruction of haemocytes caused by WSSV infection at the late stage of experiment [51]. However, the NOS activity in *M. japonicus* kept at a high level for a longer time than that of *F. chinensis* after WSSV infection. More interestingly, there was an apparent increase of LPS-stimulated NOS activity in *M. japonicus* 48 h after WSSV infection, in contrast to the relatively stable level of LPS-stimulated NOS activity in *F.chinensis*. Referred to the different sensitivity of the two species to WSSV infection, as indicated in the mortality experiment, the results on NOS activity analysis let us hypothesize that the NOS activity in haemocytes of the shrimps might have a relation to the disease resistance ability of the shrimps to WSSV infection.

Although the importance of NO and iNOS in host defense reactions against bacteria have been extensively studied in vertebrates [11], their roles in resisting virus infections is not very well defined due to the very complicated situation in virus resistance of organisms. From some previous in vitro studies, it has been demonstrated that NO could inhibit the replication of a diverse range of viruses, especially for DNA viruses, such as murine poxvirus and herpes viruses, and some RNA viruses, such as Coxsackievirus[52]. However, for virus like coronavirus, lymphocytic choriomeningitis virus and murine vaccinia virus, etc., the anti-viral effect of NO remains unclear or uncertain [52]. Besides, the overproduction of NO will affect the host defense through its effects on the balance between the type 1 helper T cell (Th1) and type 2 helper T cell (Th2), and the pathological processes through the oxidative stress it caused. Therefore, its intriguing to understand the role of NO in resistance of virus infection. For iNOS activity, it has been reported that it could be induced by many different virus in vertebrates [53–55], through the direct up-regulation of virus or the indirect effect mediated by pro-inflammatory cytokines such as interferon-γ. However, the inhibition of iNOS activity and NO production was also reported recently. It's hard to make a straightforward remark for the role of NO and iNOS in virus resistance among different organisms to the infection caused by a variety of virus. And the situation is even worse for invertebrates, although there are some reports about the invertebrate NO synthesis [56–64], none of them gave information either on NOS or NO in virus resistance. In our present study, we found that NOS activity in the haemocytes of the two species of shrimp's response differently to WSSV

infection, which is in correspondence with the different sensitivity of the two species. WSSV, reported as a large dsDNA virus belonging to the virus family *Nimaviridae*, genus *Whispovirus* [65], might be a target of NO. And the observed increase of NOS activity and LPS-induced activity in *M. japonicus* shortly after WSSV infection might play a role in resistance to virus infection, which lead to a relatively longer surviving time of the species after WSSV infection. However, all these hypotheses need to be further studied to elucidate the role of NO and NOS in host defense and pathological process in WSSV infected shrimps.

LPS have been successfully used in many studies to induce the NOS activity in haemocytes of invertebrates [17,66,29,21]. In the present study, the addition of LPS could also induce the NOS activity in haemocytes of shrimps, and the LPS-stimulated NOS activity in *M. japonicus* was much higher than that without LPS stimulation after WSSV infection. However, it still needs to be further examined that the induction of NOS activity is good or bad for the mitigation of WSSV infection. The only thing we observed was that *M. japonicacus*, with higher LPS-stimulated NOS activity after WSSV infection, could survive longer than *F. chinensis*. If the mitigation effect of increased NOS activity on WSSV infection could be confirmed, it would be helpful for the development of proper immunostimulants, and for the understanding of the mechanisms of certain immunostimulants in host defense ability against the virus infection.

In summary, our results show that the haemocytes of both species of shrimps present NOS activity after LPS stimulation. The comparison of NOS activity in the two species of shrimps after WSSV infection shows that *M. japonicus*, with higher LPS–stimulated NOS activity after WSSV infection, could survive longer than *F. chinensis*. The results suggest that NOS in haemocytes of shrimps might be involved in the non-specific defense of shrimps to WSSV infection. However, further experiments are still needed to confirm the expression of NOS in haemocytes of shrimps, and to elucidate the role of NOS in resisting the infection of WSSV virus.

Acknowledgments

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