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Short communication

Nitric oxide synthase-dependent immune response against gram negative bacteria in a crustacean, *Litopenaeus vannamei*

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ABSTRACT

Nitric oxide (NO) is a short-lived radical generated by nitric oxide synthases (NOS). NO is involved in a variety of functions in invertebrates, including host defense. In previous studies, we isolated and sequenced for the first time the NOS gene from hemocytes of *Panulirus argus*, demonstrating the inducibility of this enzyme by lipopolysaccharide *in vitro* e *in vivo*. Hyperimmune serum was obtained from rabbits immunized with a *P. argus* −NOS fragment of 31 kDa produced in *Escherichia coli*, which specifically detected the recombinant polypeptide and the endogenous NOS from lobster hemocytes by *western blotting* and immunofluorescence. In the present work, we demonstrate that the hyperimmune serum obtained against *P. argus* NOS also recognizes *Litopenaeus vannamei* NOS in hemocytes by *western blotting* and immunofluorescence. Our data also show that while the hemolymph of *L. vannamei* has a strong antibacterial activity against the Gram negative bacteria *Aeromonas hydrophila*, the administration of the anti NOS serum reduce the natural bacterial clearance. These results strongly suggest that NOS is required for the shrimp immune defense toward Gram negative bacteria. Therefore, the monitoring of induction of NOS could be an important tool for testing immunity in shrimp farming.

1. Introduction

Nowadays, many crustacean species are being commercially exploited around the world, and specially decapods. Research into the immune system of these species may provide new insights into the management and control of diseases in natural and artificial environments. Nitric oxide (NO) is a key molecule produced in the innate immune systems of many species for antimicrobial defense. However, how NO production is regulated during bacterial infection in invertebrates, especially crustaceans, remains poorly understood. Nitric oxide is produced by nitric oxide synthases (NOS), a group of evolutionarily conserved cytosolic or membrane bound isoenzymes that convert the amino acid L-arginine to citrulline and NO, which is oxidized into nonvolatile stable products, nitrite (NO_2^-) and nitrate (NO_3^-). In mammals, three isoforms of NOS have been identified. Two of these are constitutively expressed, mainly in the brain (nNOS) and endothelial cells (eNOS) and they are Ca^{2+}/calmodulin dependent. A third enzyme, the inducible Ca^{2+}-independent isoform (iNOS), is expressed in some cell types after stimulation with *Escherichia coli* lipopolysaccharide (LPS) and/or different cytokines such as interferon-γ, interleukin-1β, or tumor necrosis factor-α [1,2]. On the contrary, only one NOS gene has been reported in most invertebrate genomes. The three vertebrate NOS isozymes are presumed to have descended from a single ancestral gene in the course of invertebrate evolution [3].

The insights of the NOS/NO response in crustaceans upon PAMPs (pathogen-associated molecular patterns) challenge are still unclear. For example, while in *Panulirus argus E. coli* lipopolysaccharide (LPS) treatment stimulated both NO production and NOS mRNA in hemocytes [4,5], LPS treatment apparently increased NO production [6,7] but not NOS mRNA expression [6] in the hemocytes of *Penaeus monodon*. Moreover, although the cDNA sequences of NOS have been cloned in several shrimp species including *Marsupenaeus japonicus* [8], *Litopenaeus vannamei* [9], and *Penaeus monodon* [6], direct demonstration that NOS transcription or

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activity is correlated with bacterial clearance is virtually non-existent except for one recent report which demonstrated that endogenous NO produced by *L. vannamei* hepatopancreatic cells occurs in enzymatic regulated manners and it is sufficient to act as a bactericidal molecule for *Vibrio harveyi* clearance [10].

In previous studies, we isolated and sequenced for the first time the NOS gene from hemocytes of *P. argus* [4]. Hyperimmune serum (anti *Pa* 31 kDa-NOS) was obtained from rabbits immunized with a polypeptide of 31 kDa from *P. argus* NOS (*Pa* 31 kDa-NOS) produced in *E. coli*, which specifically detected the recombinant polypeptide and the endogenous NOS from lobster hemocytes by *western blotting* and immunofluorescence [5]. In this study, after demonstrating the recognition of *L. vannamei* NOS by anti *Pa* 31 kDa-NOS, we focused on the antimicrobial effect of NOS against the Gram negative bacteria *Aeromonas hydrophila* infection in the hemolymph of *L. vannamei*.

2. Material and methods

2.1. Experimental animals and bacterial strain

Sexually immature, healthy Pacific white shrimps (*L. vannamei*) between 8 and 10 g of weight were provided by the Yaguacan Hatchery (Cienfuegos, Cuba) and were kept in aerated seawater (35 mg/L), with controlled photoperiod of 12:12 h light:darkness until use. All experiments were performed after one week in these conditions. Experimental animals were treated according with the European Union Directive (EEC, 1986) for the protection of animals used for experimental and other scientific purposes. *A. hydrophila* used in this study was previously isolated from diseased shrimps in our laboratory.

2.2. Hemolymph collection

Hemolymph for the different assays was collected through the ventral sinus using a pyrogen-free disposable syringe containing the same volume of pre-cooled anticoagulant solution. A modified Citrate-EDTA buffer (400 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 20 mM EDTA, pH 4.6) was used as anticoagulant [11].

For hemocyte collection, the mixture was centrifuged at 700 g for 10 min at 4 °C. The cell pellet was washed twice with anticoagulant and suspended again in anticoagulant solution for *western blotting* and immunohistochemistry assays. For the antibacterial assays 20 μL of the total hemolymph was kept in individual tubes before centrifugation.

2.3. Immunodetection of native NOS in hemocytes by western blotting

Polypeptides from hemocytes were extracted using the following solution: 400 mM NaCl, 10 mM KCl and Triton 1%, pH 7.0. Samples were resolved in 10% SDS-PAGE under non-reducing conditions (1% glycerol, 0.4% SDS, 12.5 mM Tris–HCl, pH 6.6) and were electrotransferred overnight onto nitrocellulose membranes (Amersham) using a TransBlot™ transfer (BioRad). The membrane was blocked for 2 h with 5% skimmed milk (Oxoid), incubated overnight with the anti *Pa* 31 kDa-NOS serum previously obtained by us [5] diluted 1:10000 in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_47H_2O$, pH 7.3) and washed with PBST (PBS plus 0.05% Tween 20) three times for 30 min. Afterward, the membrane was incubated with a goat anti-rabbit IgG-horseradish peroxidase conjugate (Amersham) for 1 h, subsequently washed, and the enhanced chemiluminescence detection system (Amersham Biosciences) was used for visualization of protein bands.

2.4. Purification of IgG specific against P. argus NOS

Total IgG specific for *P. argus* NOS was purified following the protocol described by Gómez et al. [12]. Briefly, the Sepharose CL-4B (Amersham-Pharmacia) was moderately activated (6–12 μmol/ml) of cyanate esters with Cyanogen bromide (Merck) according to the Wilcheck classification using a modified procedure already described by Axen [13,14]. A modified Koenig reaction determination of cyanate ester concentration on the support was made [15]. The CNBr activated Sepharose CL-4B matrix was wetted in 1 mM HCL for 15 min, and washings with 0.1 M Na_2CO_3/0.1 M $NaHCO_3$-0.5 M NaCL pH 8.3 were performed. The recombinant 31 kDa *Pa*-NOS was coupled by covalent bonds on the support at pH 8.3 during 2 h at 25 °C by gentle stirring. Then, 0.1 M glycine pH 8.0 was added to block free reactive groups and five alternate washings with 0.1 M $C_2H_3O_2Na$/0.5 M NaCL pH 4.0 and 0.1 M Na_2CO_3/0.1 M $NaHCO_3$-0.5 M NaCL pH 8.3 were made. Finally, the immunosorbents were washed and stored in PBS/0.01% Tiomersal pH 7.2 at 4 °C until use [12].

The processes of antiserum binding and elution were made according to the protocol proposed by the European Molecular Biology Laboratory (http://www.embl.de). Briefly, after washing the beads 3 times with PBS in a 50 mL Corning tube, 1 mL of beads was resuspended with 10 mL of PBS and 10 mL of antiserum. The incubation was overnight at 4 °C on a tumbler. The elution of antibodies was performed at 4 °C. First, the beads were washed 3 times with 50 mL of PBS and then resuspended in 10 mL of PBS. Then, the beads were loaded into a chromatography column where they were washed with PBS for approximately 1 h until OD 280 = 0. To elute the bound antibodies, 200 mM glycine pH 2.8 were added to the column at 4 °C collecting 1 mL aliquots into tubes containing 27 μL of 3 M Tris–HCl, pH 8.8 and 100 μL of 3 M KCl. The OD 280 of fractions was measured and the antibodies solution was dialyzed at 4 °C against PBS overnight. Total proteins were quantified using the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. Aliquots were kept at −20 °C until use.

2.5. Immunodetection of native NOS in hemocytes by immunofluorescence

For indirect immunofluorescence staining, shrimp hemocytes were washed twice with anticoagulant and finally exposed *in vitro* to 0.5 mg/mL of *E. coli* LPS as previously described [5]. After 1 h the hemocytes were extended on poly-Lysine treated coverslips and then fixed in cold acetone for 10 min. Cells were then washed with PBS, blocked with BSA 3% in PBS for 30 min and incubated with the primary antibody diluted in PBS (1:400 of purified anti-*Pa* 31 kDa-NOS serum) at 37 °C for 75 min. Cells were then washed with PBS for 30 min at room temperature and incubated with anti-rabbit Alexa fluor 488 labeled secondary antibody (Molecular Probes) at 37 °C for 45 min. Finally, cells were washed twice in PBS and mounted in PBS-glycerol containing DAPI at 0.1 μg/mL. A Zeiss Axiophot microscope equipped with a 63 × NA 1.3 oil-immersion objective was routinely used. Images were taken with a SPOT Camera (Diagnostic Instruments Inc.) using the same settings, and processed with Adobe Photoshop CS6.

2.6. In vitro and in vivo hemolymph antimicrobial assay

For the *in vitro* hemolymph antimicrobial assay, *A. hydrophila* bacteria were grown in Tryptic Soy Broth. Briefly, logarithmic phase microorganism cultures were diluted in the broth to an estimated OD_{600} of 0.001, which is approximately equivalent to 10^5 colony-forming units (CFU)/mL. Diluted microorganism (90 μL) was mixed with 10 μL of medium (control) or the hemolymph in wells of a

polypropylene microtiter plates (Greiner Bio-One). Hemolymph from 3 shrimps per treatment was assayed by duplicate. The samples were 2-fold serially diluted. The growth was monitored, after an overnight incubation at 28 °C, by measuring the change in the absorbance of the culture at 600 nm using a microplate reader.

For the *in vivo* assay, two doses of *A. hydrophila* were assayed: 1×10^5 and 1×10^7 CFU. Eight shrimp per dose were injected with 50 μL solution of *A. hydrophila* or injection of saline solution alone as a negative control. Four individuals from each group were sampled at 5 and 24 h post injection (hpi) and 10 μL of pure, 1:10 and 1:100 diluted hemolymphs were plated in Luria Bertani agar plates. Plates were incubated for 24 h at 28 °C, and afterward the CFU were determined by counting.

2.7. Effect of the immuno-neutralization of NOS on NOS activity in the hemolymph of L. vannamei

Ten animals were distributed in two experimental groups of five animals per group. One group was injected with 50 μL of pre-immune serum previously obtained by us [5] and the other with the same volume of purified specific anti *Pa* 31 kDa-NOS serum. The concentration of proteins of the purified NOS antiserum and the pre-immune serum were adjusted to be equivalent (1.2 mg/mL). After 5 h post-injection, hemolymph was extracted and plasma was used to measure nitrites as an indicator of NOS activity *in vivo*. To achieve this objective nitrates were first converted to nitrites using the nitrate reductase enzyme (Boehringer, Germany). Briefly, 50 μl of samples were added to each well and mixed with 50 μl of a freshly prepared reaction mixture containing nitrate reductase (2 U/mL) (Boehringer, UK), reduced nicotinamide adenine dinucleotide phosphate (NADPH: 0.344 mM) and flavine adenine dinucleotide (FAD: 0.044 mM) in phosphate buffer. Two fold serial dilutions of sodium nitrate (250 μM) were used as standard curve. The plates were incubated at 37 °C for 1 h. Total nitrite was determined adding 200 μl of Griess reagents (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H_3PO_4). This method is based on the reaction of nitrites with Griess reagents, which generates a purple-azo dye product that can be monitored by a spectrophotometer at 540 nm.

2.8. Effect of the immuno-neutralization of NOS on the antibacterial activity of the hemolymph of L. vannamei

For this experiment, four experimental groups and five animals per group were established. Shrimps were co-injected with 50 μL containing 1×10^5 CFU of *A. hydrophila* and 50 μl of the respective treatment: Group 1: Negative control 450 mM NaCl, Group 2: L-NMMA 120 μg/mL (Sigma) (N^G-Monomethyl-L-arginine, an inhibitor selective for mammalian cNOSs), Group 3: pre-immune serum, Group 4: purified anti *Pa*31 kDa-NOS serum. The concentration of proteins of the purified NOS antiserum and the pre-immune serum were adjusted to be equivalent (1.2 mg/mL). Animals were kept in 10 L of sea water with aeration. After 24 h post-injection, hemolymph were extracted and 10 μL of pure, 1:10 and 1:100 diluted hemolymph were plated in Luria Bertani agar plates. Plates were incubated for 24 h at 28 °C. Afterward, the CFU was determined for each treatment. This experiment was performed in duplicate.

2.9. Statistical analysis

The statistical analysis was done using Graph Pad Software Inc. version 6 (San Diego, CA, USA).

3. Results and discussion

3.1. Immunodetection of native NOS from L. vannamei cells by western blotting and immunofluorescence

In order to detect the endogenous NOS from *L. vannamei*, homogenates of hemocytes were analyzed by SDS-PAGE under non-reducing conditions followed by *western blotting*. Consequently, a predominant band was obtained with the expected size of the shrimp NOS homodimer (Fig. 1A) according to the sequence reported by Yao et al. [9]. These authors reported a nucleotide

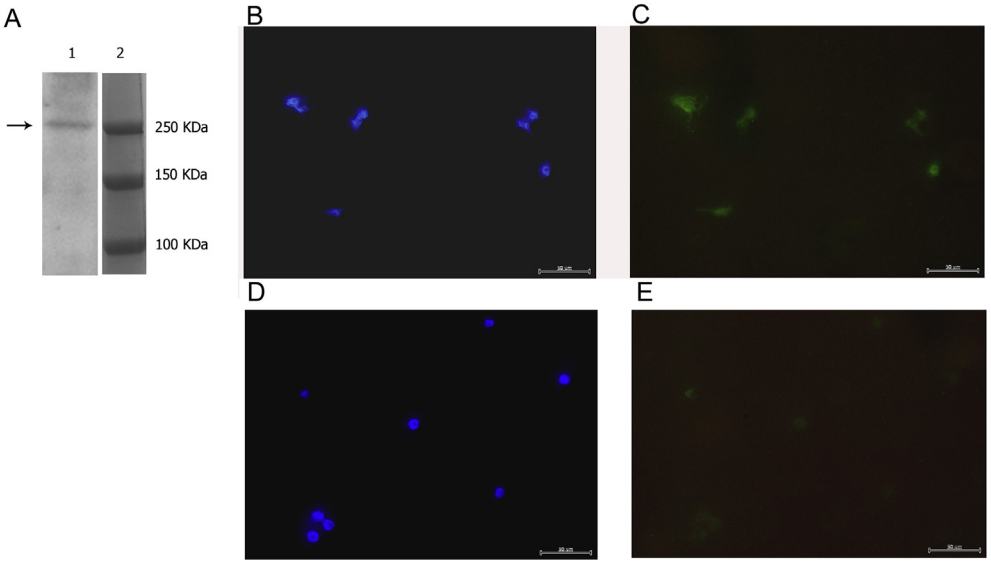

Figure 1. (A) Detection of NOS in *L. vannamei* hemocytes homogenates by *western blotting* under non reducing conditions (Lane 1). The molecular weight marker Precision Plus Protein Dual Color Standards (Biorad) was used (Lane 2). (B) Immunofluorescent localization of NOS in *L. vannamei* hemocytes. Hemocytes were incubated with rabbit anti-Pa 31 kDa-NOS serum, rabbit anti IgG Alexa fluor 488 conjugate, and DAPI for nuclear stain. The panels B and D show the staining with DAPI of hemocytes. The panel C shows the specific NOS staining of hemocytes exposed to LPS of *E. coli* 0.5 mg/mL. The panel E shows the detection of NOS in unexposed hemocytes. The images represent the immunofluorescent staining of hemocytes from six animals. A Zeiss Axiophot microscope equipped with a 63 × NA 1.3 oil-immersion objective was used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequence contains an open reading frame of 3540 bp encoding a polypeptide of 1179 amino acids with theoretical molecular weight of 133 kDa. The result obtained in the *western blotting* demonstrated the capacity of the anti-*Pa* 31 kDa-NOS antibody obtained in lobster to detect NOS in shrimps allowing us its use in further experiments. Native NOS was also specifically recognized by the anti-*Pa* 31 kDa-NOS antibody in the hemocytes using immunofluorescence microscopy. Despite that the results are qualitative; the intensity of the fluorescence seems to be higher in those cells exposed to *E. coli* LPS with respect to the unexposed hemocytes (Fig. 1B–E). Control hemocytes incubated with pre-immune serum showed no background fluorescence (data not shown). Similar results were obtained with hemocytes of the lobster *P. regius* [5], and again the treatment with LPS caused aggregation of cells and morphological changes, probably attributable to processes of cell degranulation. In addition to *P. regius* lobster and the results obtained herein, NOS has also been previously detected by immunostaining in Y-organ of the crab *Gecarcinus lateralis* [16] and *Carcinus maenas* [17] but using a universal NOS antibody. The increase in fluorescence intensity observed after LPS stimulation are in agreement with the previous finding in *L. vannamei* in which the levels of NOS transcripts sharply increased at 3-h post-injection, and were 8.8 times higher than in the control after injection with 50 μL of 0.2 mg/mL LPS from *E. coli.* These authors suggested that the *L. vannamei* NOS in hemocytes may play an important role in shrimp defense against pathogen infection [9].

3.2. In vitro and in vivo hemolymph antimicrobial assay

It is widely known that shrimps have an innate immune response involving both cellular and humoral factors and one of the main groups of pathogens affecting shrimps is Gram negative bacteria, recognized by the host immune system because of the LPS located in the outer layer of the bacterial cell wall [18,19]. In this study, we performed both in *vitro* and *in vivo* experiments to demonstrate the bacterial hemolymph clearance capacity in shrimps, specifically against *A. hydrophila* (Fig. 2). In all cases, the results showed the strong antibacterial activity of the hemolymph. The dose of 1×10^5 CFU resulted in higher clearance percent at 5 h as compared to 1×10^7 CFU ($p < 0.05$). However, the dose of 1×10^5 CFU at 24 h was selected for further experiments because in these conditions the bacterial clearance reached 100% (Fig. 2B,C). Additionally, two shrimps died at 24 h with the dose of 1×10^7 CFU. Antibacterial activity has previously been described in the hemolymph and/or the hemocytes of a wide range of crustacean species [20–23]. There are several important factors reported to be involved in this antibacterial activity such as antimicrobial peptides, lectins, phenoloxidase and nitric oxide among others [24].

3.3. Effect of the immuno-neutralization of NOS on its activity and the anti-bacterial activity of the hemolymph of L. vannamei

Shrimps injected with the anti-*Pa* 31 kDa-NOS antibody showed lower NOS activity in the hemolymph after 5 h post injection as compared with shrimps injected with pre-immune serum ($p < 0.05$) (Fig 3A). These results showed the capacity of the anti-*Pa* 31 kDa-NOS antibody to neutralize NOS activity in shrimps. After this information was obtained, shrimps were exposed *in vivo* to the Gram negative bacteria *A. hydrophila* to finally demonstrate the effect of the immunoneutralization of NOS on the bacterial clearance in the hemolymph. As a result, we obtained that after 24 h of injection with of 1×10^5 CFU of bacteria, the plates with hemolymph from shrimps injected with saline solution or pre immune serum did not show any bacterial growth (Fig. 3 B, C, D), while

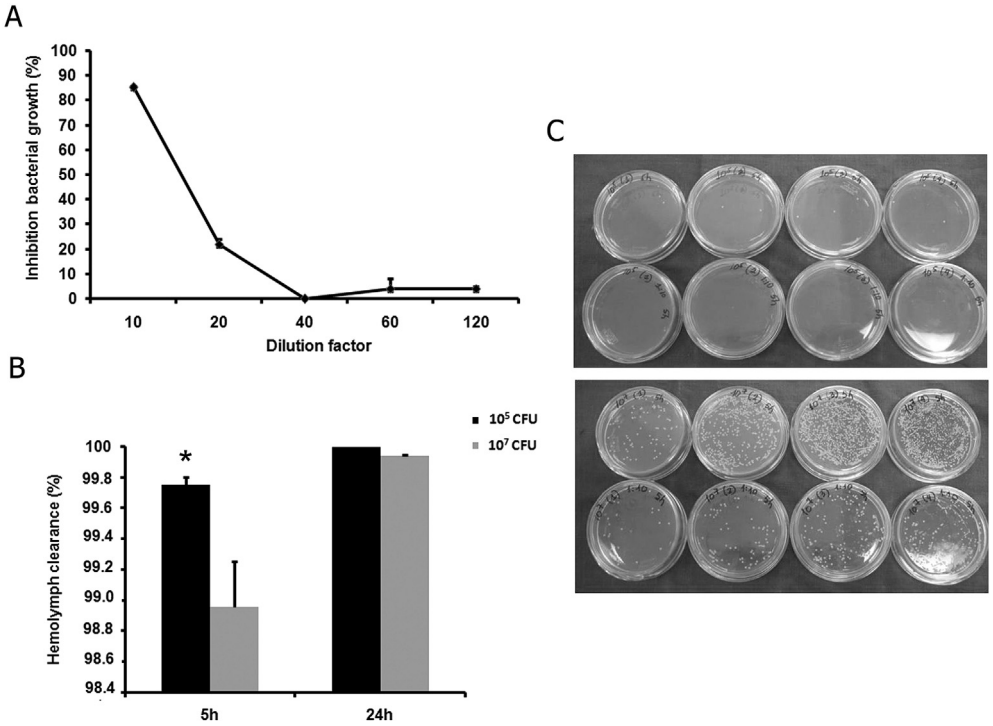

Fig. 2. *In vitro* (A) and *in vivo* (B,C) hemolymph antimicrobial assay against *Aeromonas hydrophila*. (A) The graph represents the inhibition of bacterial growth as compared to control (medium) Hemolymph from 3 shrimps was assayed by duplicate. (B) The graph represents the percent of hemolymph clearance at 5 and 24 hpi as compared to initial dose. Eight shrimp per dose (1×10^5 and 1×10^7 CFU) were assayed. Four individuals from each group were sampled at each time. Data are shown as media + standard error. A Mann Whitney test was performed because data have unequal variances (*) $p < 0.05$ (C) Agar plating in LB medium after 24 h of pure and 1:10 diluted hemolymph from 5 hpi-sampling: 1×10^5 CFU (upper panel) and 1×10^7 CFU (lower panel).

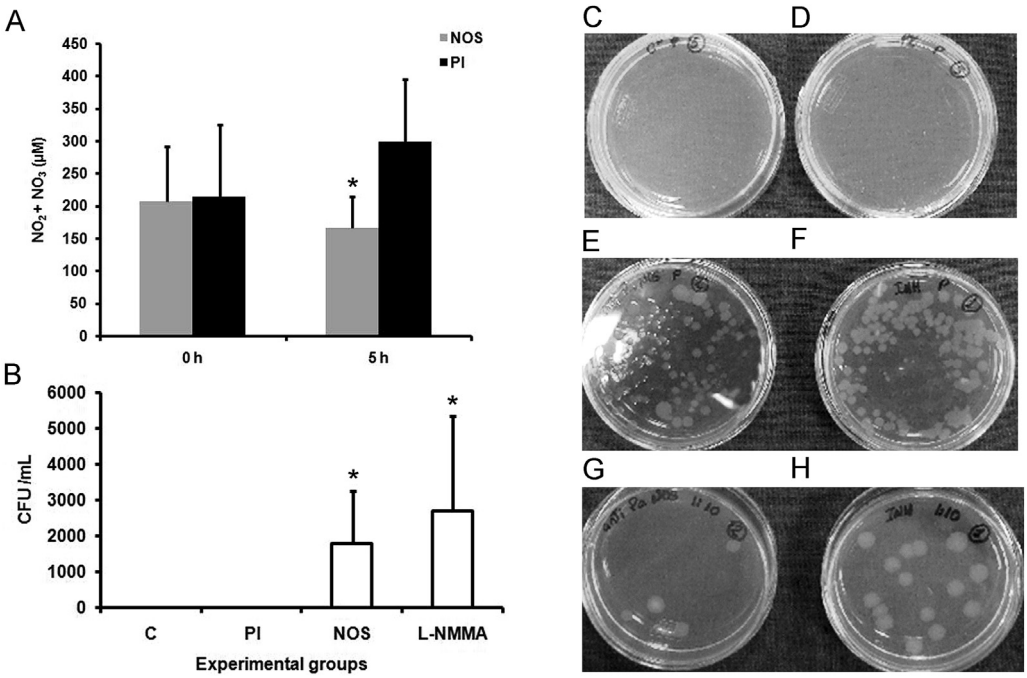

Fig. 3. (A) Effect of the immuno-neutralization of NOS on NOS activity after 5 h post-injection with purified specific anti Pa 31 kDa-NOS serum (NOS). Control group was injected with pre-immune serum (PI). Data are shown as media + standard error (N = 5). An unpaired t test was performed after transforming data. (*) $p < 0.05$ (B) Effect of the immuno-neutralization of NOS on the antibacterial activity in the hemolymph of *L. vannamei*. Shrimps were co-injected with 50 µL containing 1×10^5 CFU of *A. hydrophila* and 50 µl of the respective treatment: 450 mM NaCl(C), L-NMMA at a concentration of 120 µg/mL, pre-immune serum (PI), purified anti Pa 31 kDa- NOS serum (NOS). The graph represents data from one experiment and is representative of results from two such experiments. Data are shown as media + standard error (N = 5). An unpaired t test was performed after transforming data to asses differences between treated and its respective control (NOS vs. PI and L-NMMA vs. C). (*) $p < 0.05$. (C–H) Agar plating after 24 h: (C) Control, (D) PI (E,G) NOS, (F,H) L-NMMA. (C–F) undiluted hemolymph, (G–H) 1:10 diluted hemolymph.

plates with hemolymph from shrimps injected with the inhibitor of NOS (L-NMMA) or the anti-*Pa* 31 kDa-NOS antibody showed bacterial growth depending on the dilution of the hemolymph (Fig 3 B, E–H). The fact that we didn't detect any bacteria growth for both control groups after 24 h indicated that the hemolymph antibacterial activity wasn't affected by any component of the rabbit plasma.

L-NMMA is known as a very well established cell-permeable competitive NOS inhibitor in mammals, used to evaluate the role of the L-arginine: nitric oxide pathway (Ki values are 700 nM, 3.9 µM and 650 nM for eNOS, iNOS and nNOS, respectively). However, L-NMMA selective activity has also been demonstrated in invertebrates. For example, *E. coli* LPS increased the number of bacteria found around the immunocytes of the molluscan *Viviparus ater*, but this effect was selectively prevented by the addition of L-NMMA, suggesting that this bacterial clumping is caused by the cells liberating NO [25]. Also, the nitrite/nitrate-generating activity in homogenates of crayfish (*Procambarus clarkii*) hemocytes was inhibited by adding L-NMMA to the incubation [26].

In addition, other mammalian NOS inhibitors have been used to study NOS in invertebrates. In cultured primary shrimp hepatopancreatic cells, coincubation with L-NAME (L-NG-Nitroarginine methyl ester) ablated *V. harveyi* induced-NO production in a dose dependent manner [10]. These authors found that this NOS inhibitor enhanced the survival rate of infecting *V. harveyi* in cultured primary *L. vannamei* hepatopancreatic cells after 24 h of exposure, suggesting that NO from inducibly expressed NOS could be important for clearing invading *V. harveyi* in shrimp hepatopancreatic cells. *V. harveyi* is also a Gram negative bacterium and a serious pathogen to marine fish and invertebrates.

It is important to consider that the *P. argus* NOS fragment selected to generate the polyclonal antibody [5] has 84% identity with a similar fragment in the protein sequence from *L. vannamei*. Besides, the identity percent ranges from 41 to 84% with arthropods sequences, being the highest identity percents with crustaceans. No similarities were found with other proteins. Thus, cross-reactivity with a protein with the same function seems to be unlikely.

The induction of NOS gene expression in crustacean by bacteria or bacterial components has been previously studied *in vitro* [4,10] and *in vivo* [5,8–10]. Therefore, we suppose the injection of *A. hydrophila* probably induced the expression of NOS in the shrimps of the present study. If it was the case, the doses of L-NMMA and anti-*Pa* 31 kDa-NOS antibody were a sufficient amount to neutralize the circulating NOS before and after the induction, significant enough to cause the decrease of the antibacterial activity of the hemolymph. The antimicrobial actions of NOS have been attributed to the nitric oxide radical itself, and to various reaction products referred to as reactive nitrogen intermediates, such as NONOates, S-nitrosothiols, peroxynitrite, nitrite and nitrous acid thatcan react with structural elements, components of the replication machinery, nucleic acids, metabolic enzymes, or with virulence-associated molecules. For example, the peroxynitrites, produced by the reaction between reactive oxygen intermediates and NO radical are capable of oxidizing SH groups, lipids and DNA, and causing nitration of tyrosine residues of proteins of the microorganism [27]. Control of pathogens by NOS-derived NO can imply their death or the reduction of the microbial metabolic activity causing sufficient impairment of pathogen proliferation *in vivo* to allow clinical resolution of the disease by the immune response (for review, see Ref. [28]).

In this work, we localized for first time NOS in shrimps hemocytes using a polyclonal antibody against *P. argus* NOS by *western blotting* and immunofluorescence. Additionally, we demonstrated that this antibody was able to reduce NOS activity *in vivo*.

Afterward, we also showed that the strong antibacterial activity of the hemolymph of shrimps against Gram negative bacteria is affected because of the administration of this polyclonal antibody or a chemical NOS inhibitor (L-NMMA). These findings contribute to the knowledge about the role of NOS in the immune response of crustaceans, specifically shrimps, to Gram negative bacteria. Moreover, this study would encourage further investigation on the regulation of NOS activity in invertebrates, its expression during infections and the possibility to use as a tool for monitoring shrimp health.

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