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Short communication

Nitric oxide as an antimicrobial molecule against *Vibrio harveyi* infection in the hepatopancreas of Pacific white shrimp, *Litopenaeus vannamei*

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ABSTRACT

Nitric oxide (NO) is a key effector 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. *Vibrio harveyi*, a Gram-negative marine pathogen, is among the most prevalent and serious threats to the world's shrimp culture industry. Its virulence typically manifests itself through shrimp hepatopancreas destruction. In the current study, we found that NO generated by an *in vitro* donor system (NOC-18) could rapidly and effectively kill *V. harveyi*. In addition, injection of heat-killed *V. harveyi* increased the concentration of NO/nitrite and the mRNA expression of nitric oxide synthase (NOS) in the hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*), the commercially most significant shrimp species. Live *V. harveyi* challenge also induced NO/nitrite production and NOS gene expression in primary *L. vannamei* hepatopancreatic cells in a time- and dose-dependent manner. Co-incubation of L-NAME, an inhibitor selective for mammalian constitutive NOSs, dose-dependently blocked *V. harveyi*-induced NO/nitrite production, without affecting *V. harveyi*-induced NOS mRNA expression. Furthermore, L-NAME treatment significantly increased the survival rate of infecting *V. harveyi* in cultured primary hepatopancreatic cells of *L. vannamei*. As a whole, we have demonstrated that endogenous NO produced by *L. vannamei* hepatopancreatic cells occurs in enzymatically regulated manners and is sufficient to act as a bactericidal molecule for *V. harveyi* clearance.

1. Introduction

Innate immunity, an ancient host defense machinery reliant on broad-spectrum antimicrobial effector activities, has been found in virtually all classes of plants and animals including vertebrates and invertebrates [1]. Nitric oxide (NO) is among the best known of such effector molecules, whose diverse effects in inflammation and innate immune response are linked to NO reactivity with transition metals via metal-nitrosyl coordination, and cysteine thiols via *S*-nitrosylation [2]. In contrast to NO's broad substrate scope, NO biosynthesis by nitric oxide synthase (NOS) is tightly controlled, which proceeds from L-arginine metabolism, and ends with NO being oxidized into a nonvolatile stable product, nitrite (NO_2^-). In mammals, three different isozymes of NOS, namely eNOS (endothelial), nNOS (neuronal) and iNOS (inducible), have been identified and extensively characterized [3]. The calcium-dependent eNOS and nNOS are constitutively expressed in many tissues including heart and brain, while iNOS is inducible by microbial products or inflammatory cytokines in various cell types such as monocytes, macrophages, endothelium and hepatocytes [4]. In stark contrast, 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 [5]. This difference in gene expression between invertebrate and vertebrate NOS raises the

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intriguing question whether the invertebrate NOS has its own distinct patterns of regulation both in transcription and enzymatic activity.

Compared to highly reactive oxygen species (hROS) such as hypochlorous acid and hydroxyl radical, whose biological targets are primarily amino acids and nucleic acids, respectively, NO shows only low to moderate reactivity towards most biomolecules and its antimicrobial activity is quite poorly understood [6]. In mammals, macrophages and other myeloid-derived phagocytes produce large amounts of NO via iNOS in response to infectious agents including bacteria, viruses, parasites and fungi [6,7]. In the presence of concomitant superoxide production from NADPH oxidase (NOX), NO can give rise to the potent reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$) [8], which exhibits generally very high antimicrobial activity through nucleic acid oxidation, protein nitration, and lipid peroxidation [2]. However, a good handful of pathogenic bacteria have been reported to show resistance to nitrosative stress exerted by RNS produced by host [9]. Indeed, Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* produces NO from their own bacterial NOS, while other bacteria produce NO through nitrite/nitrate reductases. Given this context, it is interesting to ask whether the antimicrobial activity of NO is generalizable across all bacterial species, and whether NO generation in invertebrate cells is efficient enough to sustain bactericidal actions.

So far, there is a paucity of research on NO's antimicrobial activity during bacterial infection in invertebrates. In crustaceans, although cDNA sequences of NOS have been cloned in several shrimp species including *Litopenaeus vannamei* [10], *Marsupenaeus japonicus* [11] and *Penaeus monodon* [12], direct demonstration that NOS transcription or activity is correlated with bacterial clearance is virtually non-existent. Moreover, the nature of NOS/NO-mediated immune response in crustaceans upon PAMPs (pathogen-associated molecular patterns) challenge remains controversial. For example, *Escherichia coli* lipopolysaccharide (LPS) treatment reportedly triggered NO production [12,13] but not NOS mRNA expression [12] in the hemocytes of *P. monodon*. On the other hand, *Vibrio penaeicida* and LPS challenge stimulated NOS mRNA synthesis in the gill of *M. japonicus* [11] and the hemocytes of lobster *Panulirus argus* [14,15], respectively.

In this study, we focused on NO's role in invertebrate immune response to bacterial infections in shrimp as a marine crustacean model. Pacific white shrimp (*L. vannamei*) is commercially the most significant shrimp species, whose cultivation has periodically suffered enormous losses due to vibriosis [16]. The Gram-negative bacterium *Vibrio harveyi* is a serious pathogen to marine fish and invertebrates, particularly, penaeid shrimp [17]. It is recognized as a common causative agent of vibriosis in *L. vannamei*, which can take the form of "bacterial white tail disease" [18] or "bright-red syndrome" [19]. In crustaceans, the digestive gland hepatopancreas is one of the major sites for vibrio infection, which often leads to necrosis of hepatopancreatic cells and atrophy of hepatopancreatic tubules [20,21]. Hepatopancreas is reportedly the tissue with the highest expression level of NOS mRNA in *L. vannamei* [10]. In terms

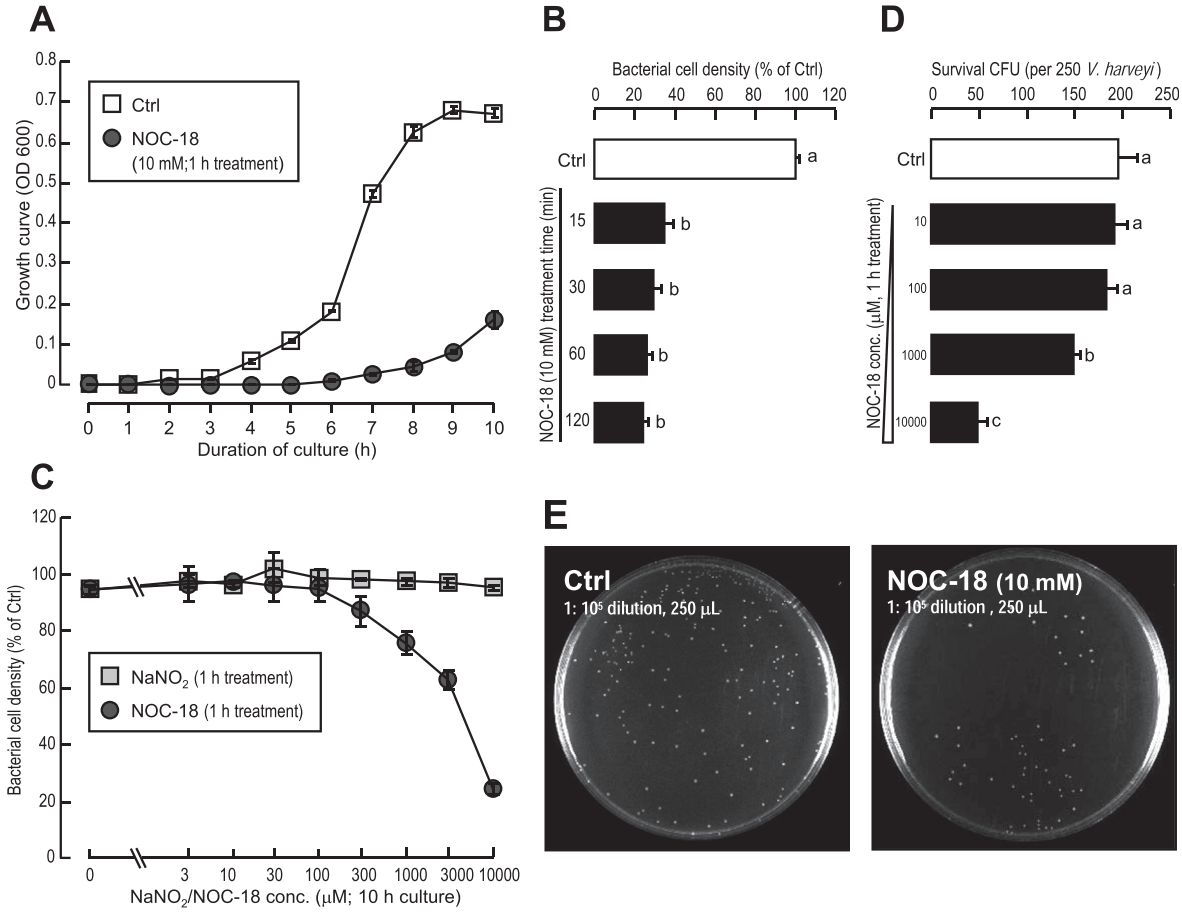

Fig. 1. Effects of NO (NOC-18) on growth and survival of *V. harveyi*. A: comparison of *V. harveyi* growth curves with or without NO treatment; B: cell densities of *V. harveyi* grew in 2216E for 10 h after NO treatment for different durations; C: cell densities of *V. harveyi* grew in 2216E for 10 h after NO treatment with different concentrations; D: CFU of survival *V. harveyi* after NO treatment with different concentrations; E: agar plating of *V. harveyi* with or without NO treatment.

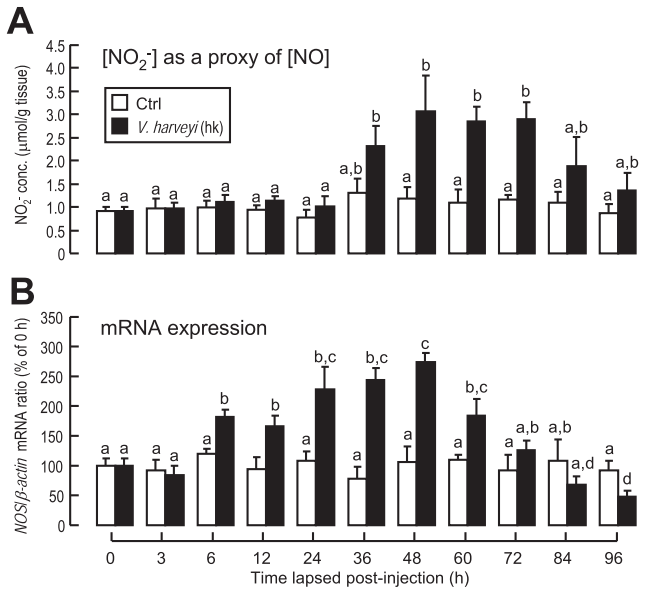

Fig. 2. A: NO/nitrite production in the shrimp hepatopancreas after *V. harveyi* challenge; B: NOS mRNA expression in the shrimp hepatopancreas after *V. harveyi* challenge.

of bactericidal activity against vibrio species, however, little is known about how NO production is regulated in hepatopancreas of *L. vannamei*, despite the fact that the transcriptional expression of hepatopancreatic NOS was found to be significantly increased after vibrio challenge in another crustacean species, the mud crab *Scylla paramamosain* [22].

In this study, we focused on the antimicrobial effect of NO against *V. harveyi* infection in the hepatopancreas of *L. vannamei*. We studied the kinetics and dose-dependent effects of NO killing of *V. harveyi* by using NOC-18, a highly stable chemical donor that releases NO in aqueous solutions without requiring co-factors and without producing toxic metabolites [23]. We also monitored the changes of hepatopancreatic NO/nitrite concentration and NOS mRNA level after *V. harveyi* challenge. Furthermore, the effects of an established mammalian cell-permeable NOS inhibitor (L-NAME, which have K_i values of 15 nM, 39 nM, and 4.4 μM for nNOS, eNOS and iNOS, respectively [24], and can be hydrolyzed into the fully functional inhibitor, L-NNA, by cellular esterases [25]) on bacterially induced-NO/nitrite production and *V. harveyi* survival were examined.

2. Materials and methods

2.1. Experimental animals and bacterial strain

Sexually immature, healthy Pacific white shrimps (*L. vannamei*) with body weight of 11.3 ± 0.8 g and body length of 9.7 ± 0.9 cm were collected from the Dongfang shrimp culture center, Zhanjiang, Guangdong, China. Shrimps were anaesthetized on ice and sacrificed by decapitation. Hepatopancreatic samples for *in vivo* experiments were collected and rapidly frozen in liquid nitrogen, followed by storage at −80 °C prior to further analysis. All animal experiments were conducted in accordance with the guidelines and approval of the Ethics Committees of South China Sea Institute of Oceanology, Chinese Academy of Sciences.

Vibrio harveyi (CCTCC number: AB2010412) used in this study was previously isolated from diseased shrimps in our laboratory. The *V. harveyi* strain was grown on 2216e marine agar or broth with

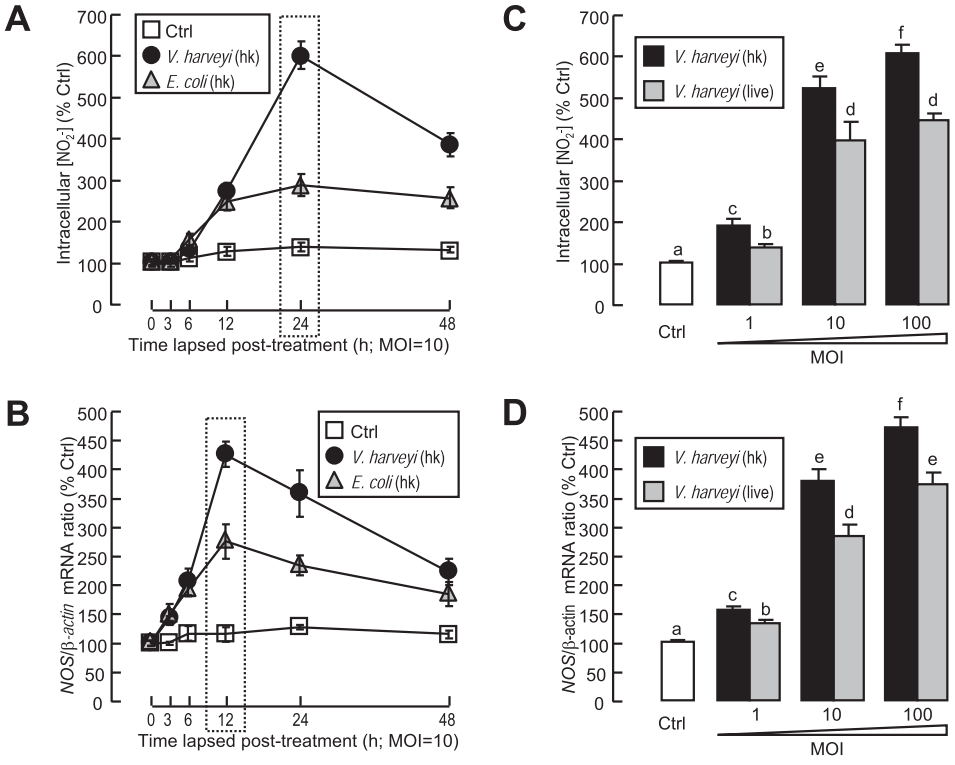

Fig. 3. Effects of heat-killed or live *V. harveyi* challenge on NO/nitrite production and NOS mRNA expression in cultured primary shrimp hepatopancreatic cells. A: time-dependent effects of *V. harveyi* challenge on NO/nitrite production; B: time-dependent effect of *V. harveyi* challenge on NO/nitrite production; C: dose-dependent effects of *V. harveyi* challenge on NOS mRNA expression; D: dose-dependent effects of *V. harveyi* challenge on NOS mRNA expression.

33‰ salinity at 30 °C. Counts of colony-forming units (CFU) of *V. harveyi* culture were determined by measuring their optical density (OD) at 600 nm, against a standard curve constructed with our previous plating results for serial dilution (1.0 OD corresponds to 1.7×10^9 CFU/mL).

2.2. Growth and survival assay of V. harveyi

V. harveyi was cultured in 2216e broth at 30 °C until $OD_{600\ nm}$ reached 0.6 and harvested by centrifugation at 3000 × *g* for 5 min. The collected bacterial cell pellet was washed three times in phosphate buffered solution (PBS) with 33‰ salinity. Re-suspended cells were diluted to a density of 1.0×10^8 CFU/mL and statically incubated in PBS (33‰ salinity) containing NO or nitrite (NO_2^-) at 30 °C. NO was generated from a chemical donor NOC-18 (Sigma) and NO_2^- was prepared as aqueous $NaNO_2$. Solid NOC-18 powder was dissolved in PBS at pH 7.4 and 37 °C to give a 20 mM stock solution for assays. After drug incubation, the cells were then harvested, washed three times in PBS (33‰ salinity) and re-suspend in 1 mL 2216e broth. Re-suspend cells of 10 μL with 1: 10^5 dilution were added into 4 mL 2216e broth and then incubated at 30 °C with shaking at 220 rpm. A parallel plating assay for each sample was also performed by using 250 μL diluted cells plated onto 2216e agar. To test the effect of NO on *V. harveyi* growth, the NOC-18 dose and treatment time were fixed to 10,000 nM and 1 h, respectively, and $OD_{600\ nm}$ values were measured from 0 to 10 h after the start of culture. For kinetics study of NO effects on *V. harveyi* survival, the NOC-18 dose was fixed to 10,000 nM with different durations of NO challenge. To evaluate the dose-dependent effects of NO on *V. harveyi* survival, treatment time was fixed to 1 h. The $OD_{600\ nm}$ values were measured at 10 h after the start of culture in both kinetics and dose-dependency studies.

2.3. In vivo challenge of Pacific white shrimp with V. harveyi

Heat-killed *V. harveyi* was diluted in sterile artificial seawater (33‰ salinity) with a density of 1.0×10^8 CFU/ml. Each shrimp was injected with 100 μL solution of *V. harveyi* and injection of artificial seawater alone was used as a negative control. Three individuals of hepatopancreatic samples from each group were randomly sampled at 0–96 h post injection (hpi).

2.4. Isolation, static cultures and V. harveyi challenge of shrimp hepatopancreatic cells

Shrimp hepatopancreatic cells were freshly prepared as described previously [26] and cultured in DMEM/F-12 (Gibco BRL) with 5% fetal bovine serum (FBS) in 24-well polyethylenimine (PEI, Sigma) pre-coated plates at 30 °C with 5% CO_2 at a final density of 2.0×10^5 cells/mL. After 18-h culture for recovery, the culture medium was replaced with serum-free DMEM/F-12 with bacteria being added at an appropriate multiplicity of infection (MOI) and/or test substances at indicated concentrations. To test the maximal level of cumulative NO/nitrite induction and NOS mRNA expression after bacterial challenge, heat-killed *V. harveyi* or *E. coli* (JM109) at MOI of 10 were added to the culture medium, and shrimp hepatopancreatic cells were collected at different time points after challenge. A comparative study was performed between live and heat-killed *V. harveyi*, with the bacteriostatic chloramphencol (5 μg/mL) being routinely added to culture medium in place of penicillin (100 U/mL). In NOS inhibitor tests, solid L-NAME (Sigma) powder was dissolved in DMSO to a stock concentration of 50 mM and further used by diluting in culture medium. In these cases, the bacterial MOI was fixed at 10, and the duration of bacterial challenge and inhibitor treatment was fixed at 24 h and 12 h for detection of NO/nitrite production and NOS mRNA expression, respectively.

2.5. Measurement of NO production and NOS mRNA expression

NO production in shrimp hepatopancreatic tissue and cultured primary cells was estimated by spectrophotometrically measuring the levels of nitrite as a proxy of NO with a Griess reagent system (Promega). Briefly, 0.5 g of tissue samples were homogenized by sonication in 2.5 mL PBS on ice, and cell samples were lysed by repeated freezing and thawing after wash (2 ×) with PBS. Samples were then centrifuged at 10,000 × *g* for 10 min at 4 °C to separate the soluble component and cell debris. To measure nitrite content, 50 μL of the supernatant was incubated with 50 μL of sulfanilamide solution at room temperature for 10 min. Next, 50 μL of NED solution was added to each sample and incubated for another 10 min. Absorbance was measured at $OD_{540\ nm}$ by using a microplate reader (Thermo Scientific). Nitrite content for each sample was calculated based on a standard curve constructed with aqueous $NaNO_2$.

Total RNA from tissue samples and primary culture cells was isolated by using TRIzol reagent (Invitrogen), digested with DNase I (Invitrogen), and reverse transcribed with Superscript® II (Invitrogen). Transcriptional expression of lv-NOS and β-actin (as an internal control) was detected by using SYBR Premix Ex Taq™ II (TaKaRa) in a RotorGene RG-3000 (Qiagen) with primers and PCR conditions described in supplementary data 1. Serially diluted plasmids containing lvNOS and β-actin sequences were used as standards for real-time PCR. The raw data of NOS expression in

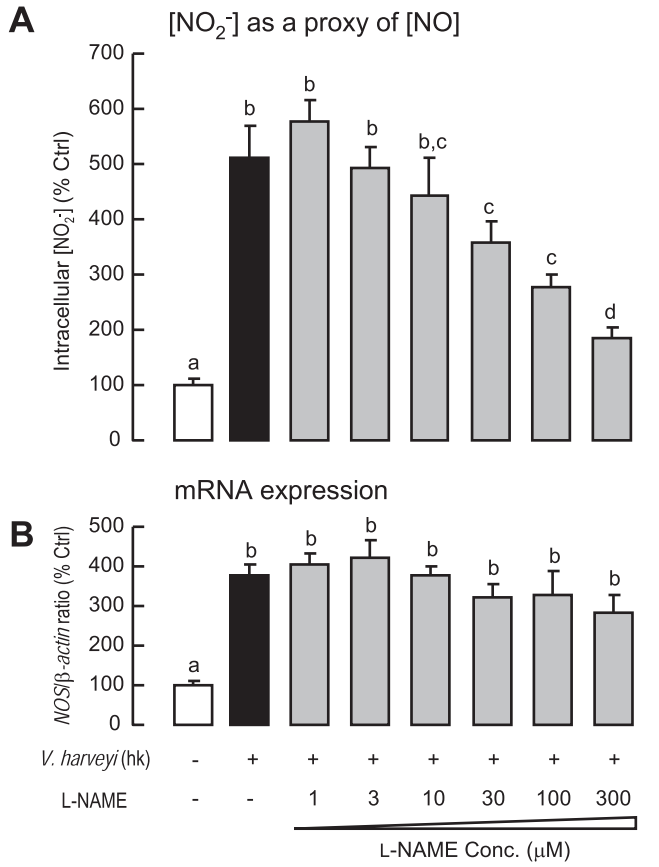

Fig. 4. A: Dose-dependent effects of L-NAME (NOS inhibitor) on *V. harveyi*-induced NO/nitrite production in cultured primary shrimp hepatopancreatic cells; B: dose-dependent effects of L-NAME (NOS inhibitor) on *V. harveyi*-induced NOS mRNA expression in cultured primary shrimp hepatopancreatic cells.

terms of fmol target transcript detected per tube were routinely normalized as a ratio of β-actin mRNA detected in the same sample.

2.6. Survival of *V. harveyi* in cultured primary shrimp hepatopancreatic cells

Survival assay of *V. harveyi* in cultured primary shrimp hepatopancreatic cells following pharmacological inhibition of NOS was performed by modification of the macrophage invasion assays [27]. Briefly, live *V. harveyi* grown to mid-log phase were added to primary culture at an MOI of 10. After incubation at 30 °C for 1 h, infected hepatopancreatic cells were washed twice with PBS, and DMEM/F-12 containing gentamicin (50 μg/mL) was then added to kill any remaining extracellular bacteria. After 2 h of further incubation at 30 °C, fresh DMEM/F-12 with gentamicin (50 μg/mL) was replaced again. At the end of infection (22 h later), infected hepatopancreatic cells were washed twice with PBS and *V. harveyi* were harvested by lysing the host cells with 500 μL of 0.1% Triton X-100 (3 min). Cell lysates were collected and diluted 100,000 × in PBS, and aliquots in triplicates were plated onto 2216E agar to assess CFU count.

2.7. Data transformation and statistical analysis

Raw data in this study were simply transformed as a percentage of mean values of the untreated control group for statistical analysis. Data expressed as mean ± SEM were analyzed by using Student's *t*-test or one-way ANOVA followed by Fisher's least significant difference (LSD) test by using SPSS (IBM Software). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Effects of NO on V. harveyi growth and survival

After static incubation with NO (generated by its donor NOC-18) for 1 h, turbidity of the *V. harveyi* culture was visibly diminished in comparison to untreated bacteria grown for the same recovery period (Fig. 1A), indicating that brief NO treatment was sufficient to inhibit *V. harveyi* growth. Under NOC-18 treatment at 10,000 nM, the first 15 min seemed especially important for inhibition of NO on *V. harveyi* growth. NO treatment for just 15 min could achieve an 86% inhibitory effect of 2 h treatment on *V. harveyi* growth (Fig. 1B). The inhibitory effects of NO on *V. harveyi* growth occurred in an dose-dependent manner, whereas its metabolic end product NO_2^- did not alter *V. harveyi* growth when used in the same range of molar concentrations (Fig. 1C), suggesting that the antibacterial effects of NO on *V. harveyi* was mediated by NO itself or its secondary products (such as $ONOO^-$) but not NO_2^-. Agar plating results (Fig. 1D and E) further support that NO inhibited *V. harveyi* growth by killing a substantial portion of the inoculating *V. harveyi* cells through irreversible nitrosative stress, since removing NOC-18 at the end of the assay did not result in a rebound of bacterial growth. NO treatment was also effective in killing *V. harveyi* in a dose-dependant manner, as reflected in a marked decrease in surviving CFU count in the agar plates. Although NO and its secondary products may exert toxic effects on microbes, microbial pathogens have evolved a number of mechanisms to cope with such host RNS [9]. Previous studies have demonstrated the NO tolerance in several microbes such as *E. coli* [28,29], *Campylobacter jejuni* [30] and *Rhodobacter capsulatus* [31]. Based on *in vitro* data as shown in Fig. 1, *V. harveyi* was showed sensitivity to NO as an antimicrobial molecule. It is reasonable to speculate that crustaceans (such as shrimp) known to express NOS may produce NO purposely against vibrio infection.

3.2. In vivo and In vitro induction of NO production and NOS expression following V. harveyi challenge

By *in vivo* injection of heat-killed *V. harveyi* in the hepatopancreas of *L. vannamei*, NO/nitrite production and NOS mRNA expression were found to be increased in an inducible manner. A significant surge in NO/nitrite was observed from 36 h onwards post-infection, with the maximal response seen at 48 h after challenge (Fig. 2A). In parallel, stimulated expression of NOS mRNA was observed from 6 h after challenge, which peaked at 48 h after challenge (Fig. 2B). In primary culture of *L. vannamei* hepatopancreatic cells, heated-killed *V. harveyi* induced NO/nitrite production in a time- (Fig. 3A, maximal response at 24 h after challenge) and dose-dependent manner (Fig. 3C, maximal response

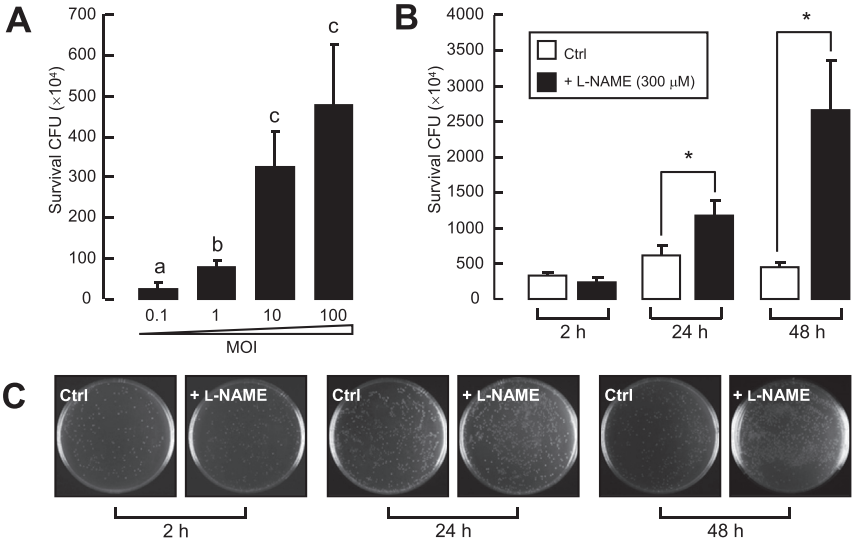

Fig. 5. A: Quantification of intracellular *V. harveyi* survival in shrimp hepatopancreatic cells with different MOI; B: quantification of intracellular *V. harveyi* survival in shrimp hepatopancreatic cells with or without co-incubation of L-NAME to block NO production. C: agar plating of *V. harveyi* in survival assay with or without co-incubation of L-NAME to block NO production.

at MOI = 100). Likewise, heat-killed *V. harveyi* also induced NOS transcriptional expression in a time- (Fig. 3B, maximal response at 12 h after challenge) and dose-dependent manner (Fig. 3D, maximal response with MOI = 100). Heat-killed *E. coli* was also found to induce NO/nitrite production and NOS expression in ways broadly similar to that of *V. harveyi* challenge (Fig. 3A and B), albeit less potently at the same MOI used. When live *V. harveyi* was used as an infectious agent in the presence of chloramphenicol (bacteriostatic), *L. vannamei* hepatopancreatic cells gave responses in NO production and NOS mRNA expression comparable to that in heat-killed *V. harveyi*, suggesting that viability of the infecting vibrio is a nonessential requirement for NO induction. Similarly, it was previously reported that increased NO production could be triggered by CpG oligodeoxynucleotides (ODNs, a kind of PAMPs) treatment in HEK293T cells transfected with shrimp Toll-like receptor [32]. However, the nature of immunostimulatory bacterial molecules and the mechanisms underlying receptor recognition and signal transduction involved need to be further validated. Our study here provides evidence that NO production and NOS expression can to a large extent be upregulated in response to vibrio challenge, suggesting that NOS in shrimp is inducible in nature. As only one NOS gene has been reported in invertebrates, we reason that the NOS may be both constitutively and inducible expressed in shrimp, in manners distinct from isozymes found in vertebrates [5]. Based on analysis of the above data (Figs. 2 and 3), we have demonstrated that *V. harveyi* is a potent stimulant for inducing NO production and NOS mRNA expression in the hepatopancreas of *L. vannamei*.

3.3. Inhibition of NO production limits hepatopancreatic cells' capacity to V. harveyi

In cultured primary shrimp hepatopancreatic cells, co-incubation with L-NAME (constitutive NOS-selective inhibitor in mammals) ablated *V. harveyi* induced-NO production in a dose-dependent manner (Fig. 4A), without any effect on *V. harveyi* induced-NOS mRNA expression (Fig. 4B). This result indicates that L-NAME inhibiting NO production only at the NOS activity level and the NO levels do not feedback on NOS mRNA expression in an autocrine manner. Similar blocking of NO production by mammalian NOS inhibitors has previously been reported in the haemocytes of *P. monodon* [12,33]. Additionally, L-NAME treatment strongly enhanced the survival rate of infecting *V. harveyi* in cultured primary *L. vannamei* hepatopancreatic cells. As shown in Fig. 5A, survival of *V. harveyi* was positively associated with infection MOI. As expected, survival of *V. harveyi* was significantly higher in the presence of NOS inhibitor at 24 h and 48 h post-infection (Fig. 5B), suggesting that NO from inducibly expressed NOS could be important for clearing invading *V. harveyi* in shrimp hepatopancreatic cells.

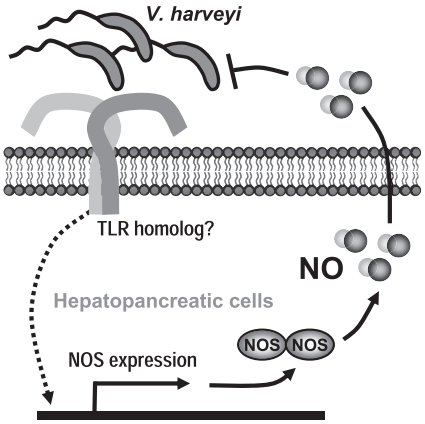

Fig. 6. Working model for NO as an antimicrobial molecule against *V. harveyi* infection in *L. vannamei* hepatopancreas.

In conclusion, our study has demonstrated the following: (1) NO inhibits growth of *V. harveyi* by rapidly and effectively killing *V. harveyi*; (2) *V. harveyi* challenge potently induces NO production and NOS mRNA expression in the shrimp hepatopancreas; (3) NOS inhibitor (L-NAME) blocks lvNOS activity and reduces shrimp hepatopancreatic cells' capacity for NO-dependent vibrio clearance. Collectively, we have here provided evidence that NO is a potentially important antimicrobial molecule against *V. harveyi* infection in *L. vannamei* hepatopancreas *in vitro* and *in vivo* (Fig. 6). We think that this study would encourage further investigation on the regulation of invertebrate NOS activity and expression in infections and shed light on NO's antimicrobial effects towards overlooked yet emerging pathogens such as vibrios.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2014.10.042.

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