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Mutual modulation between norepinephrine and nitric oxide in haemocytes during the mollusc immune response

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Nitric oxide (NO) is one of the most important immune molecules in innate immunity of invertebrates, and it can be regulated by norepinephrine in ascidian haemocytes. In the present study, the mutual modulation and underlying mechanism between norepinephrine and NO were explored in haemocytes of the scallop *Chlamys farreri*. After lipopolysaccharide stimulation, NO production increased to a significant level at 24 h, and norepinephrine concentration rose to remarkable levels at 3 h and 12~48 h. A significant decrease of NO production was observed in the haemocytes concomitantly stimulated with lipopolysaccharide and α-adrenoceptor agonist, while a dramatic increase of NO production was observed in the haemocytes incubated with lipopolysaccharide and β-adrenoceptor agonist. Meanwhile, the concentration of cyclic adenosine monophosphate (cAMP) decreased significantly in the haemocytes treated by lipopolysaccharide and α/β-adrenoceptor agonist, while the content of Ca^{2+} was elevated in those triggered by lipopolysaccharide and β-adrenoceptor agonist. When the haemocytes was incubated with NO donor, norepinephrine concentration was significantly enhanced during 1~24 h. Collectively, these results suggested that norepinephrine exerted varied effects on NO production at different immune stages via a novel α/β-adrenoceptor-cAMP/Ca^{2+} regulatory pattern, and NO might have a feedback effect on the synthesis of norepinephrine in the scallop haemocytes.

Nitric oxide (NO) is an important signaling molecule that is involved in a broad range of physiological processes[1–3], and plays an indispensable and crucial role in immune defense of vertebrates[4–6]. After infection by a pathogen, high amounts of NO can be synthesized in the immunocytes of the host to directly eliminate the invasive pathogen or induce inflammation[7–9]. Recently, NO is also reported to be implicated in the immune response of invertebrates[7,10]. For example, massive NO production has been observed during the haemocyte-mediated melanotic encapsulation of *Drosophila*[11], and NO is also found to be involved in the immunomodulation of several mollusc species, including scallop, clam, mussel, snail and oyster[12–17]. NO synthesized in the haemocytes could modulate their phagocytosis and apoptosis in scallop, and regulate the antibacterial activity and redox homeostasis of the haemolymph[18].

High concentration of NO is of strong cytotoxicity during the immune response, and it can not only kill the invasive pathogens but also hurt the normal host cells[19–22]. For example, glucocorticoid and estrogen promoted proinflammatory responses and elicited cell damages at the same time, which were triggered by high level of inducible NO in mammals[23,24]. Therefore, the rigorous modulation of NO concentration is essential for the maintenance of immune homeostasis and other normal physiological processes. The neuroregulation of NO is one of the most important regulatory pathways to prevent the hosts from exaggerated or prolonged immune response. For instance, the brain-derived neurotrophic factor (BDNF) could protect cultured cortical neurons from NO-elicited glutamate neurotoxicity[25]. But the neuroendocrine modulation of NO was seldom reported in invertebrates. As an important neuroendocrine hormone, catecholamine was able to modulate the level of NO in the ascidian haemocytes[26]. And the NO production could be modulated by the cell signal pathways containing protein kinase A (PKA), extracellular signal-regulated kinase (ERK) and protein kinase C (PKC) in mollusc[13,27]. However, the exact

signaling pathways of catecholamine modulation on NO and the regulatory mechanisms remain unclear in mollusc.

Catecholamines act as neurotransmitter and hormone in catecholaminergic neuroendocrine system, and immunomodulator in immune system in vertebrate[28]. Catecholamines are synthesized from the catecholaminergic neuroendocrine system and released into serum during the immune stress[28,29]. Catecholamines can be *de novo* synthesized in the immunocytes and secreted in an autocrine or a paracrine manner[30]. These released catecholamines can couple with the adrenoceptors (ARs) on the surface of immunocytes[31], and subsequently regulate the intracellular level of specific second messengers and then trigger certain signaling pathway to modulate the immune response[32]. It has also been reported that catecholamines can serve as an important immunomodulator in invertebrates[33]. For example, norepinephrine (NE), epinephrine and AR antagonist could negatively modulate the immune response against bacteria challenge in scallop *Chlamys farreri*[34]. NE could also regulate the level of reactive oxygen species (ROS) and phagocytosis of haemocytes via β-AR in oyster *Crassostrea gigas*[35,36], as well as a range of immunological activities in Sydney rock oyster *Saccostrea glomerata*, including phenoloxidase and acid phosphatase activities, phagocytic activity, and superoxide and peroxide production[37].

The scallop *C. farreri* is one of the most important economic maricultural bivalves. In recent years, the industry of scallop aquaculture has suffered from severe diseases, leading to massive mortality and grievous loss. Investigations of NE modulation on NO would contribute to the understanding of the immune defense mechanism of scallop and hopefully lay a foundation for the prevention or control of diseases. The purposes of this study were (1) to shed a light on the response of NE and NO against lipopolysaccharide (LPS) stimulation, (2) to explore the impact of exogenous NO on NE concentration in scallop haemocytes, (3) to investigate the possible regulatory mechanism of immunological NE on NO production, including the specific binding with counterpart receptors and the activation of cyclic adenosine monophosphate (cAMP) or Ca^{2+}, for better understanding of the mutual communications between neuroendocrine and immune systems in mollusc.

Results

The temporal change of NO and NE concentration in haemocytes after LPS stimulation. The NO production and NE concentration in the cultured haemocytes both increased significantly after LPS stimulation. The production of NO increased to a significant level at 24 h after LPS stimulation (Fig. 1). NE concentration ascended twice after LPS stimulation compared with that in the control group. The first increase appeared at 3 h, and the second rise extended from 12 to 48 h and peaked at 48 h after LPS stimulation (Fig. 2).

NO production in response to the stimulation of AR agonists and antagonists with/without LPS. After incubation only with AR agonists or antagonists at 18°C for 24 h, respectively, the basic level of NO did not change significantly in the haemocytes (Fig. 3). However, after LPS stimulation, NO concentration was significantly elevated in comparison with that in the control group ($P < 0.05$), whereas declined to the basal level after the co-incubation of LPS with phenylephrine. On the contrary, there was an increase in NO production induced by LPS and isoproterenol stimulation than that induced by single stimulation of LPS. There were no significant changes in the concentration of NO after the co-stimulations of LPS and prazosin or propranolol.

The change of cAMP concentration after the stimulation of LPS, LPS and AR agonists or antagonists. Single stimulation of LPS or concomitant stimulation of LPS with prazosin or propranolol did not induce the change of the cAMP level in scallop haemocytes. However, after co-stimulation of LPS with phenylephrine and isoproterenol, the concentration of cAMP was significantly reduced in comparison with control group (Fig. 4).

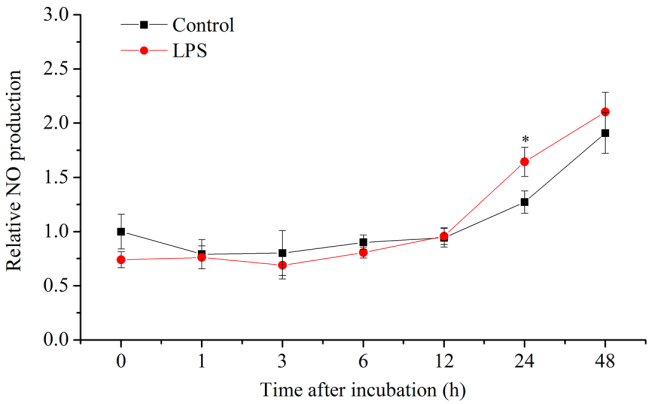

Figure 1 | Nitric oxide (NO) production after the *in vitro* stimulation of *Chlamys farreri* haemocytes with LPS. Control: L-15; LPS: 5 μg mL^{-1} in L-15. Data presented as mean ± S.D. (N = 6) of folds of control NO production at 0 h. The significant differences among the control and treated groups were subjected to multivariate analysis based on general linear model. *Asterisks* indicated significant differences ($P < 0.05$) between LPS-treated and control groups at single time points.

The change of Ca^{2+} content after the stimulation of LPS, LPS and AR agonists or antagonists. After LPS stimulation, Ca^{2+} concentration was significantly increased compared with that in the control group ($P < 0.01$). Co-stimulation of LPS with phenylephrine, prazosin or propranolol did not elicit any change of Ca^{2+} concentration compared with the LPS-induced Ca^{2+} level, while the stimulation of LPS and isoproterenol dramatically enriched Ca^{2+} content comparing the single stimulation of LPS (Fig. 5).

The effect of SNP on NE concentration in scallop haemocytes. After the stimulation of SNP, the concentration of NE quickly reached the summit at 1 h, and kept significantly higher levels from 3 to 24 h in comparison with that in the control group. And then, NE concentration restored to the basic level at 48 h with no significant difference in comparison with that in the control group (Fig. 6).

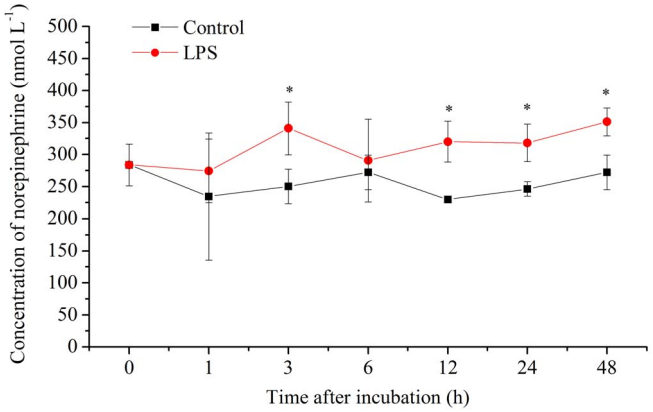

Figure 2 | Norepinephrine (NE) concentration after *in vitro* stimulation of *C. farreri* haemocytes with LPS. Control: L-15; LPS: 5 μg mL^{-1} in L-15. Data presented as mean ± S.D. (N = 6) of NE concentration expressed as nmol L^{-1}. The significant differences among the control and treated groups were subjected to multivariate analysis based on general linear model. *Asterisks* indicated significant differences ($P < 0.05$) between LPS-treated and control groups at single time points.

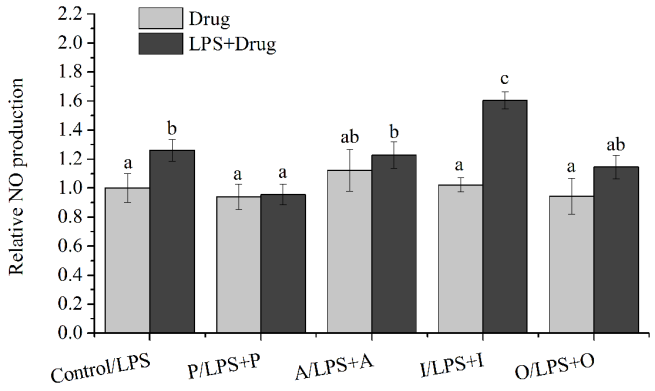

Figure 3 | Relative NO production at 24 h after *in vitro* haemocyte stimulation with agonists and antagonists of adrenoceptors (ARs) with/without LPS. Control: L-15; P: phenylephrine; LPS+P: LPS+phenylephrine; A: prazosin; LPS+A: LPS+prazosin; I: isoproterenol; LPS+I: LPS+isoproterenol; O: propranolol; LPS+O: LPS+propranolol. Data presented as mean ± S.D. (N = 6) of folds of control NO production at 0 h. The significant differences among the control and treated groups were subjected to one-way ANOVA, followed by Student-Newman-Keuls multiple range test, which revealed three groups (a to c). Relative NO production with the same letter in common do not differ at the $P = 0.05$ level of significance.

Discussion

Both of vertebrates and invertebrates primarily rely on the functional integration of neuroendocrine and immune systems to survive from exogenous or endogenous threats[31]. Though fundamental elements of a primordial 'neuroendocrine system' were suggested to be present in the Bilaterian ancestor[38], exact properties and signaling pathways in invertebrates and their differences with vertebrates are still far from well-understood. In addition, the knowledge on the interaction pattern of neuroendocrine and immune system in invertebrates remains unclear and incomplete. The present study focused on the reciprocity between catecholamines and immune system in scallop *C. farreri* to elucidate the possible regulatory loops within neuroendocrine and immune system of mollusc.

The *in vivo* response of NO production to LPS stimulation and the immunomodulation of NO have been previously reported in scallop *C. farreri*[18]. In the present study, the concentrations of NO and NE were measured in the primary cultured scallop haemocytes after LPS stimulation. The results demonstrated that NO production in haemocytes was induced significantly at 24 h post LPS stimulation, and NE concentration reached a significantly higher level at 3 h and retained higher level from 12 to 48 h. The induction of NE after LPS stimulation further evidenced the presence of *de novo* synthesis of NE in the fully armed scallop haemocytes, which has been reported in haemolymph of *Mytilus galloprovincialis* Lmk[39,40]. On the other hand, the changes in concentration of NO and NE indicated their involvement in scallop immune system, and the potential role of NE in regulating early local immune response[41]. When NE concentration ascended the second time to a stable higher level from 12 to 48 h, NO production exerted a transient increase, intimating that NE might act to modulate NO production during the immune response induced by LPS. In conclusion, the transitory change of NO and rapid increase of NE revealed the capability of scallop immunocytes to synthesize NE *de novo*, the involvement of both NO and NE in immune response against LPS, as well as the potential role of NE in modulating LPS-induced NO production.

Historically, ARs were divided into two major types termed as α- and β-AR, and NE exerted varied modulation effects on immune system when coupling with these two different types of AR on the surface of immunocytes[42,43]. In order to verify the effect of NE on NO in the response against LPS and to identify the regulatory pattern, the concentration of NO in the primary cultured scallop haemocytes was detected after the stimulation of α/β-AR agonists and antagonists with/without LPS. As a result, LPS elicited a significant increase in NO production compared with that in control group, whereas the addition of α-AR agonist phenylephrine decreased the concentration of NO, while the addition of β-AR agonist isoproterenol, on the contrary, dramatically elevated the NO level. These results verified the modulation effect of NE on NO production. Particularly, it was suggested that NE attenuated the production of NO when coupling with α-AR, whereas facilitated the production of NO when coupling with β-AR during the immune response against LPS. Further, when comparing the expression time courses of NO and NE after LPS stimulation, it might be speculated that NE bonded with α-AR to inhibit the generation of NO during early phases of immune defense, whereas it bonded with β-AR and induced higher level of NO production in later phases. However, in acute immune responses of ascidian haemocytes, NE down-regulated NO production by coupling with either α- or β-AR[26]. The differed results might be partly explained by the species specificity and the disparate intracellular reactions in response to different stimuli. However, the determinants of NE binding affinity with α- and β-AR, as well as its preference in certain immune response in mollusc, are far from well-understood. Altogether, the present results suggested that NE might act discriminatively on the modulation of NO production via activating distinct types of AR at different stages of immune response against LPS. The underlying mechanisms upon the selective binding of NE with α- or

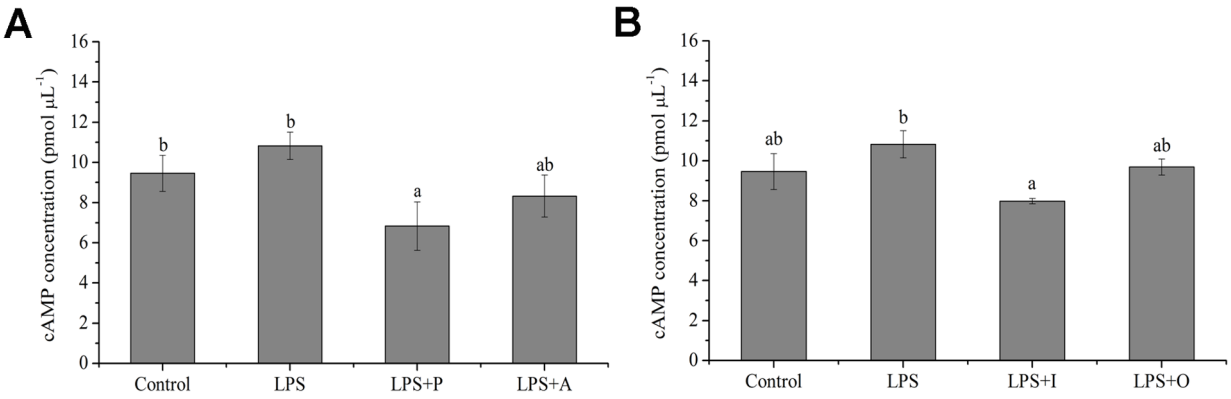

Figure 4 | Concentration of cAMP in response to *in vitro* haemocyte stimulation with LPS and α-AR (A) or β-AR (B) agonist/antagonist at 24 h. Data presented as mean ± S.D. (N = 6) of cAMP concentration expressed as pmol μL^{-1}. The significant differences among the control and treatedgroups were subjected to one-way ANOVA, followed by Student-Newman-Keuls multiple range test, which revealed two groups (a to b). The concentration of cAMP with the same letter in common do not differ at the $P = 0.05$ level of significance.

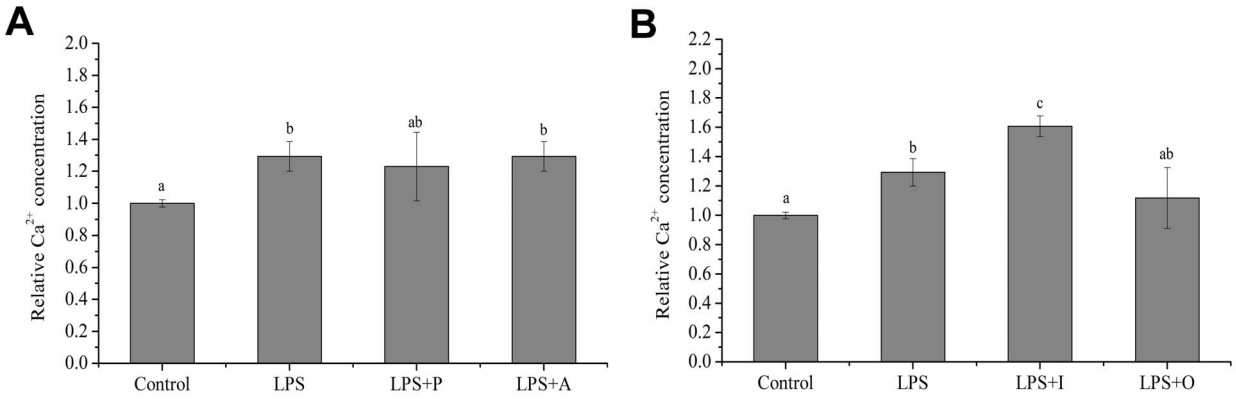

Figure 5 | Concentration of Ca^{2+} in response to *in vitro* haemocyte stimulation with LPS and α-AR (A) or β-AR (B) agonist/antagonist at 24 h. Data presented as mean ± S.D. (N = 6) of folds of control Ca^{2+} content. The significant differences among the control and treated groups were subjected to one-way ANOVA, followed by Student-Newman-Keuls multiple range test, which revealed two (A) (a to b) or three groups (B) (a to c). The relative Ca^{2+} concentration with the same letter in common do not differ at the $P = 0.05$ level of significance.

β-AR, and the corresponding second messenger systems need further investigation.

To shed light on the downstream signaling pathway of AR regulation on NO, the regulatory pattern of AR subtypes upon cAMP and Ca^{2+} were examined by detecting their concentrations after stimulations of AR agonists or antagonists with/without LPS. The level of cAMP did not change with the induction of LPS, but was significantly reduced when the scallop haemocytes were concomitantly stimulated by α- or β-AR agonist with LPS. Conversely, the content of Ca^{2+} was substantially increased after the stimulation of LPS, and rose to a drastically higher level after additional treatment of β-AR agonist. These results indicated that the activation of α-AR could down-regulate the concentration of cAMP during the immune response, while the activation of β-AR cast down the production of cAMP, whereas up-regulated the level of Ca^{2+} content. The activating pattern of the second messengers by the ARs and the following effects on immune responses in scallop are different from those in other species. According to earlier studies in vertebrates, α_1- and α_2-AR enhanced immune responses via raising Ca^{2+} content and reducing cAMP concentration, respectively[44], while β-AR hampered the immune responses by promoting cAMP generation[45]. Analysis of the adrenergic-like octopamine receptors in insects and other molluscs revealed resembled second messenger-modulating systems[46]. In short, the current report presented a novel AR-cAMP/Ca^{2+} modulating system, through which NE modulated the production of NO in the immune response of scallop.

In order to explore the feedback loops between the immune response and NE, the effect of NO on the NE concentration in scallop haemocytes was also examined in the present study. After the stimulation with SNP (NO generator), NE retained a higher concentration than basal level from 1 to 24 h, indicating the long-term up-regulation of NO on NE production. Combined with the result that NE maintained a significantly higher level during 24∼48 h after the response of NO at 24 h elicited by LPS, it was suggested that NO might regulate the level of NE concentration in scallop haemocytes during the immune response. Since NE at low concentration (<1 μmol L^{-1}) was more prone to modulate immune behaviors of immunocytes[47], NO production might have been triggered in the innate immunity to constantly promote and preserve low levels of NE below 1 μmol L^{-1}, thus to enhance the immune resistance to intrusive pathogens. In addition, the up-regulation of NO on NE concentration might have promoted a cascade of events starting with sGC activation[48,49]. However, the exact process of the feedback pathways and the molecular mechanisms need further investigations.

In summary, the present study depicted the mutual modulation between NE and NO during the immune response of scallop haemocytes against LPS (Fig. 7). NE was involved in the modulation of NO generation, while in reverse, NO might also play a role in feedback regulation of NE concentration. During the immune response against LPS, NE down-regulated NO production at initial stage of the immune response via the activation of α-AR and inhibition in cAMP expression, and up-regulated NO production at later stages through the activation of β-AR and mediation on both cAMP and Ca^{2+}. However, the exact mechanisms of NO induction by NE, including the downstream signals following the regulation of the second messengers, remain so rudimentary. Further research correlated with the interactions of NO system and other members in catecholamine system is also needed to access a more comprehensive understanding of the crosstalk between neuroendocrine and immune systems in mollusc.

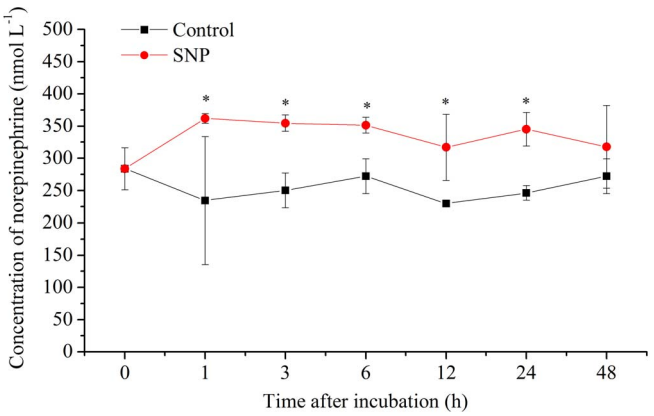

Figure 6 | Effect of *in vitro* stimulation of *C. farreri* haemocytes with SNP on NE concentration. Control: L-15; SNP: 5 mmol L^{-1} in L-15. Data presented as mean ± S.D. (N = 6) of NE concentration expressed as nmol L^{-1}. The significant differences among the control and treated groups were subjected to multivariate analysis based on general linear model. *Asterisks* indicated significant differences ($P < 0.05$) between LPS-treated and control groups at single time points.

Methods

Scallop haemocytes collection and primary haemocytes culture. Haemolymph and haemocytes were prepared as described previously by Hughes *et al*[50] with some modifications. Haemolymph was aspirated by a syringe from the adductor muscle of scallop in ALS (Alseve) buffer (115.5 mmol L^{-1} glucose, 31.0 mmol L^{-1} sodium citrate, 11.5 mmol L^{-1} EDTA and 385.0 mmol L^{-1} NaCl, pH 7.0 and 1000 mOsmol) with the ratio of 1 : 1. The suspension was centrifuged at 800 × g for 10 min and the cell pellets were resuspended in modified Leibovitz L-15 medium (supplemented with 345.7 mmol L^{-1} NaCl, 7.2 mmol L^{-1} KCl, 5.4 mmol L^{-1} $CaCl_2$, 9.0 mmol L^{-1}

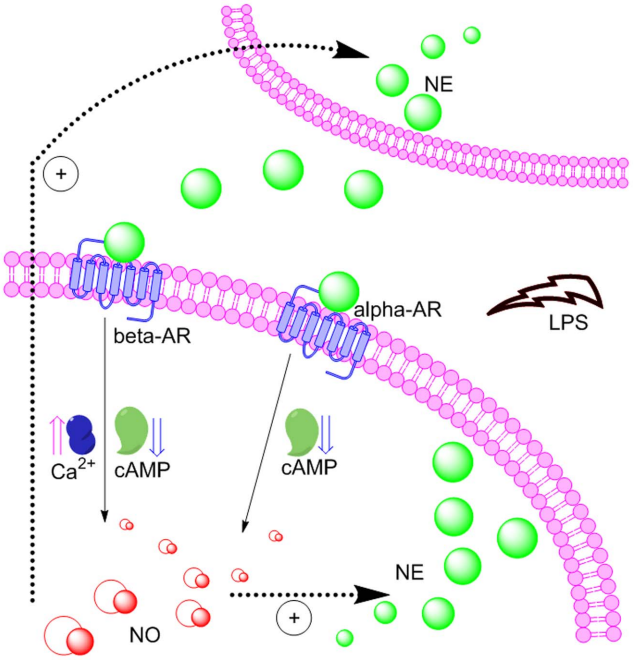

Figure 7 | Schema of signal pathways in present study. NE was secreted by autocrine or paracrine from *C. farreri* haemocytes in response to LPS, and suppressed LPS-induced NO production by binding with α/β-AR and modulating the concentration of cAMP or Ca^{2+}. NO could in turn promote the synthesis of NE.

$MgSO_4$, 41.0 mmol L^{-1} $MgCl_2$, 115.5 mmol L^{-1} glucose, 10% FCS, 299.1 μmol L^{-1} penicillin G, 171.9 μmol L^{-1} streptomycin, 83.8 μmol L^{-1} gentamicin and 0.11 μmol L^{-1} amphotericin B at pH 7.0 and 1000 mOsm), which was sterilized by filtering through 0.22 μm filters and kept at 4°C before use. The resuspended haemocytes were counted and diluted to a concentration of 8×10^5 cells mL^{-1}. After a three-day culture, the cells were recounted and adjusted to 8×10^5 cells mL^{-1} with L-15. Then 100 μL (8×10^4 cells) of cell suspension was planted into each well of the 96-well plate. The cell viability was detected by the Trypan blue exclusion technique using an assay kit (Beyotime biotechnology, China).

LPS and SNP stimulation. The cultured haemocytes were incubated with L-15 (as control group), 5 μg mL^{-1} LPS (Sigma Aldrich, USA) in L-15, and 5 mmol L^{-1} Sodium Nitroferricyanide (III) Dihydrate (SNP, Beyotime biotechnology, China) in L-15, respectively. After incubated at 18°C for 0, 1, 3, 6, 12, 24 and 48 h, the haemocytes from the control and stimulation groups were collected for the following detection of NO and NE. The trials were repeated six times.

The concomitant stimulation of adrenoceptor agonists or antagonists with LPS. The cultured haemocytes were incubated with the following stimulators including phenylephrine (α-AR agonist, 1 μmol L^{-1}, Sigma Aldrich, USA), prazosin (α-AR antagonist, 10 μmol L^{-1}, Sigma Aldrich, USA), isoproterenol (β-AR agonist, 1 μmol L^{-1}, Sigma Aldrich, USA), and propranolol (β-AR antagonist, 10 μmol L^{-1}, Sigma Aldrich, USA). The concentrations of the stimulators were referenced as described by Blais V *et al*[46]. Another set of haemocytes were concomitantly incubated with LPS (5 μg mL^{-1}) and phenylephrine (1 μmol L^{-1}), prazosin (10 μmol L^{-1}), isoproterenol (1 μmol L^{-1}) and propranolol (10 μmol L^{-1}), respectively. The incubation of haemocytes with L-15 medium was employed as the control group. After incubation at 18°C for 24 h, the haemocytes from each group were sampled for the detection of NO, cAMP and Ca^{2+}. The trials were performed for sextuples and each of them was performed in duplicate in three different assays.

Detection of NO production. NO production in the primary cultured haemocytes from the stimulation and control groups was detected spectrophotometrically by using DAF-FM DA (Beyotime biotechnology, China), the fluorescence probe of NO[51]. After the haemocytes were incubated with DAF-FM DA (5 μmol L^{-1}) in dark at 18°C for 20 min, the supernatant was discarded and the cells were washed twice and collected in steriled PBS (136.89 mmol L^{-1} NaCl, 2.68 mmol L^{-1} KCl, 8.10 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, pH 7.4). The fluorescence was detected by fluorescence spectrophotometer (HITACHI, Tokyo, Japan) with excitation wavelength of 495 nm and emission wavelength of 515 nm. Relative production of NO was calculated by comparing the control-subtracted fluorescence value of stimulated cells with that of control (N = 6).

Quantification of NE concentration. The concentration of NE in the haemocyte lysates was quantified by Norepinephrine ELISA Kit (Abnova, USA). Briefly, NE was extracted from samples using a cis-diol-specific affinity gel, then acylated and derivatized enzymatically. The derivatized standards, samples and the solid phase bound analytes competed for a fixed number of NE-antibody binding sites. After the system was equilibrated, free NE and free NE-antibody complexes were removed by washing with Wash Buffer for three times. The antibody bound to the solid phase was detected by using an anti-rabbit IgG-peroxidase conjugate with TMB as a substrate. The reaction was monitored by a microtiter plate reader (BioTek, USA) at 450 nm. Quantification of samples was achieved by comparing their absorbance with a reference curve and expressed as nmol L^{-1} (N = 6).

Measurement of cAMP concentration. The concentration of cAMP was measured following the instruction of cAMP Direct Immunoassay Kit (Abcam, Cambridge, UK). The cultured haemocytes were scraped and dissociated completely, centrifuged at 14,000 × g for 10 min to collect the supernatant as the testing sample. After neutralized and acetylated with Acetylating Reagent and Neutralizing Buffer, respectively, 50 μL of standard cAMP (or testing samples was added to the Protein G coated 96-well plate and incubated with 10 μL of cAMP antibody at room temperature for 1 h with gentle agitation. Then 10 μL of cAMP-HRP was added, and the plates were incubated for another hour. The suspension was discarded and the haemocytes in the wells were washed with 1× cAMP Assay Buffer for five times. The detecting reaction was conducted by incubating the haemocytes with 100 μL of HRP for 1 h and stopped by adding 100 μL of 1 mol L^{-1} HCl. Then the reaction was checked by the microtiter plate reader at 450 nm. The absorbance of the substrate was also detected as background absorbance and subtracted from all standards and samples. The molar concentration of cAMP in cell pellets was determined from standard curves generated using standard preparation (with final concentration of 0, 0.00078, 0.00156, 0.00312, 0.00625, 0.0125, 0.025, 0.05, 0.1 and 0.2 pmol μL^{-1}, respectively) (N = 6).

Determination of Ca^{2+} level. Ca^{2+} levels in stimulation and control groups were examined by detecting the fluorescence of Fluo-3 AM (Beyotime biotechnology, China), the fluorescence probe of Ca^{2+}. The control and stimulated haemocytes were incubated with Fluo-3 AM (5 μmol L^{-1}) in dark at 18°C for 20 min. After the supernatant was withdrew, the left cell pellets were washed with steriled PBS, and their fluorescence were detected by fluorescence spectrophotometer (HITACHI, Tokyo, Japan) with excitation wavelength of 488 nm and emission wavelength of 525 nm. Relative level of Ca^{2+} was calculated by comparing the control-subtracted fluorescence value of stimulated cells with that of control (N = 6).

Statistical analysis. All the data were expressed as mean ± S.D. (N = 6). The homogeneity of variances was checked with Levene's test and the significant differences among the control and stimulation groups were subjected to multivariate analysis (general linear model) or one-way analysis of variance (one-way ANOVA) followed by Student-Newman-Keuls multiple range test. Statistically significant difference was designated at $P < 0.05$.

1. Borsch, D. M., Cilento, E. V. & Reilly, F. D. Role for nitric oxide (NO) and not PGI-2 in acetylcholine (ACH)-induced relaxation of cremaster 3A arterioles. *FASEB J* **8**, A1034 (1994).
2. Figueroa, X. F., Gonzalez, D. R., Martinez, A. D., Duran, W. N. & Boric, M. P. ACh-induced endothelial NO synthase translocation, NO release and vasodilatation in the hamster microcirculation *in vivo*. *J PHYSIOL-LONDON* **544**, 883–896, doi:10.1113/jphysiol.2002.021972 (2002).
3. Paduch, R. & Kandefer-Szerszen, M. Transforming growth factor-beta 1 (TGF-beta 1) and acetylcholine (ACh) alter nitric oxide (NO) and interleukin-1 beta (IL-1 beta) secretion in human colon adenocarcinoma cells. *IN VITRO CELL DEV-AN* **45**, 543–550, doi:10.1007/s11626-009-9220-7 (2009).
4. Nguyen, T. *et al.* DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *PROC NATL ACAD SCI USA* **89**, 3030–3034 (1992).
5. Bogdan, C. Nitric oxide and the immune response. *NAT IMMUNOL* **2**, 907–916 (2001).
6. Sharma, D. *et al.* Differential activation of NF-[kappa]B and nitric oxide in lymphocytes regulates *in vitro* and *in vivo* radiosensitivity. *MUTAT RES-GEN TOX EN* **703**, 149–157, doi:10.1016/j.mrgentox.2010.08.010 (2010).
7. Franchini, A., Fontanili, P. & Ottaviani, E. Invertebrate immunocytes: relationship between phagocytosis and nitric oxide production. *COMP BIOCHEM PHYS B* **110**, 403–407, doi:10.1016/0305-0491(94)00163-o (1995).
8. Fang, F. C. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *NAT REV MICRO* **2**, 820–832 (2004).
9. Boscá, L., Zeini, M., Través, P. G. & Hortelano, S. Nitric oxide and cell viability in inflammatory cells: a role for NO in macrophage function and fate. *TOXICOLOGY* **208**, 249–258, doi:10.1016/j.tox.2004.11.035 (2005).
10. Rivero, A. Nitric oxide: an antiparasitic molecule of invertebrates. *TRENDS PARASITOL* **22**, 219–225, doi:10.1016/j.pt.2006.02.014 (2006).
11. Nappi, A. Nitric oxide involvement in *Drosophila* immunity. *NITRIC OXIDE* **4**, 423–430, doi:10.1006/niox.2000.0294 (2000).
12. Jeffroy, F. & Paillard, C. Involvement of nitric oxide in the *in vitro* interaction between Manila clam, *Ruditapes philippinarum*, hemocytes and the bacterium *Vibrio tapetis*. *FISH SHELLFISH IMMUN*, doi:10.1016/j.fsi.2011.10.008 (2011).

13. Wright, B., Lacchini, A., Davies, A. & Walker, A. Regulation of nitric oxide production in snail (*Lymnaea stagnalis*) defence cells: a role for PKC and ERK signalling pathways. *BIOL CELL* **98**, 265–278 (2006).
14. Novas, A., Cao, A., Barcia, R. & Ramos-Martinez, J. I. Nitric oxide release by hemocytes of the mussel *Mytilus galloprovincialis* Lmk was provoked by interleukin-2 but not by lipopolysaccharide. *INT J BIOCHEM CELL BIOL* **36**, 390–394, doi:10.1016/s1357-2725(03)00212-7 (2004).
15. Conte, A. & Ottaviani, E. Nitric oxide synthase activity in molluscan hemocytes. *FEBS LETT* **365**, 120–124, doi:10.1016/0014-5793(95)00439-g (1995).
16. Ottaviani, E., Paeman, L. R., Cadet, P. & Stefano, G. B. Evidence for nitric oxide production and utilization as a bacteriocidal agent by invertebrate immunocytes. *EUR J PHARMACOL* **248**, 319–324, doi:10.1016/0926-6917(93)90006-c (1993).
17. Villamil, L., Gómez-León, J. & Gómez-Chiarri, M. Role of nitric oxide in the defenses of *Crassostrea virginica* to experimental infection with the protozoan parasite *Perkinsus marinus*. *DEV COMP IMMUNOL* **31**, 968–977 (2007).
18. Jiang, Q. *et al.* The immunomodulation of inducible nitric oxide in scallop Chlamys farreri. *FISH SHELLFISH IMMUN* (2012).
19. Moncada, S., Palmer, R. M. & Higgs, E. A. Nitric oxide: physiology, pathophysiology, and pharmacology. *PHARMACOL REV* **43**, 109–142 (1991).
20. Estrada, C., Gómez, C., Martin, C., Moncada, S. & Gonzalez, C. Nitric oxide mediates tumor necrosis factor-α cytotoxicity in endothelial cells. *BIOCHEM BIOPH RES CO* **186**, 475–482, doi:10.1016/s0006-291x(05)80832-0 (1992).
21. Hibbs Jr, J. *et al.* Evidence for cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. *J CLIN INVEST* **89**, 867 (1992).
22. Orucevic, A. & Lala, P. K. Role of nitric oxide in IL-2 therapy-induced capillary leak syndrome. *CANCER METAST REV* **17**, 127–142, doi:10.1023/a,1005969024182 (1998).
23. Dai, R., Phillips, R. A., Karpuzoglu, E., Khan, D. & Ahmed, S. A. Estrogen regulates transcription factors STAT-1 and NF-kappa B to promote inducible nitric oxide synthase and inflammatory responses. *J IMMUNOL* **183**, 6998–7005, doi:10.4049/jimmunol.0901737 (2009).
24. Palmer, R., Bridge, L., Foxwell, N. & Moncada, S. The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *BRIT J PHARMACOL* **105**, 11 (1992).
25. Kume, T. *et al.* BDNF prevents NO mediated glutamate cytotoxicity in cultured cortical neurons. *BRAIN RES* **756**, 200–204, doi:10.1016/s0006-8993(97)00195-9 (1997).
26. de Barros, C. M., de Abreu Mello, A. & Allodi, S. Norepinephrine depresses the nitric oxide production in the ascidian hemocytes. *J INVERTEBR PATHOL* **111**, 182–185, doi:10.1016/j.jip.2012.07.002 (2012).
27. Barcia, R. & Ramos-Martinez, J. Effects of interleukin-2 on nitric oxide production in molluscan innate immunity. *INV SURV J* **5**, 43–49 (2008).
28. Romero, M. L. & Butler, L. K. Endocrinology of stress. *INT J COMP PSYCHOL* **20** (2007).
29. Perry, S. F. & Capaldo, A. The autonomic nervous system and chromaffin tissue: Neuroendocrine regulation of catecholamine secretion in non-mammalian vertebrates. *AUTON NEUROSCI* **165**, 54–66, doi:10.1016/j.autneu.2010.04.006 (2011).
30. Qiu, Y.-H., Cheng, C., Dai, L. & Peng, Y.-P. Effect of endogenous catecholamines in lymphocytes on lymphocyte function. *J NEUROIMMUNOL* **167**, 45–52, doi:10.1016/j.jneuroim.2005.06.007 (2005).
31. Ottaviani, E. & Franceschi, C. The invertebrate phagocytic immunocyte: clues to a common evolution of immune and neuroendocrine systems. *IMMUNOL TODAY* **18**, 169–174, doi:10.1016/s0167-5699(97)84663-4 (1997).
32. Kohm, A. P. & Sanders, V. M. Norepinephrine: a messenger from the brain to the immune system. *IMMUNOL TODAY* **21**, 539–542 (2000).
33. Pani, A. K. & Croll, R. P. Catechol Concentrations in the hemolymph of the scallop, *Placopecten magellanicus*. *GEN COMP ENDOCR* **118**, 48–56 (2000).
34. Zhou, Z. *et al.* The modulation of catecholamines to the immune response against bacteria *Vibrio anguillarum* challenge in scallop *Chlamys farreri*. *FISH SHELLFISH IMMUN* **31**, 1065–1071, doi:10.1016/j.fsi.2011.09.009 (2011).
35. Lacoste, A., Malham, S. K., Cueff, A. & Poulet, S. A. Noradrenaline modulates oyster hemocyte phagocytosis via a β-Adrenergic receptor–cAMP signaling pathway. *GEN COMP ENDOCR* **122**, 252–259, doi:10.1006/gcen.2001.7643 (2001).
36. Lacoste, A., Malham, S. K., Cueff, A. & Poulet, S. A. Noradrenaline modulates hemocyte reactive oxygen species production via β-adrenergic receptors in the oyster *Crassostrea gigas*. *DEV COMP IMMUNOL* **25**, 285–289, doi:10.1016/s0145-305x(00)00067-7 (2001).
37. Aladaileh, S., Nair, S. V. & Raftos, D. A. Effects of noradrenaline on immunological activity in Sydney rock oysters. *DEV COMP IMMUNOL* **32**, 627–636, doi:10.1016/j.dci.2007.10.001 (2008).
38. Hartenstein, V. The neuroendocrine system of invertebrates: a developmental and evolutionary perspective. *J ENDOCRINOL* **190**, 555–570, doi:10.1677/joe.1.06964 (2006).
39. Cao, A., Ramos-Martínez, J.-I. & Barcia, R. In hemocytes from *Mytilus galloprovincialis* Lmk., treatment with corticotropin or growth factors conditions catecholamine release. *INT IMMUNOPHARMACOL* **7**, 1395–1402, doi:10.1016/j.intimp.2007.07.008 (2007).
40. Gonzalez-Riopedre, M., Barcia, R. & Ramos-Martínez, J. I. Implication of PKC isozymes in the release of biogenic amines by mussel hemocytes: effect of PDGF, IL-2, and LPS. *J EXP ZOOL PART A* **311A**, 727–734, doi:10.1002/jez.566 (2009).
41. Di Comite, G., Grazia Sabbadini, M., Corti, A., Rovere-Querini, P. & Manfredi, A. A. Conversation galante: How the immune and the neuroendocrine systems talk to each other. *AUTOIMMUN REV* **7**, 23–29, doi:10.1016/j.autrev.2007.03.004 (2007).
42. Spengler, R. N., Chensue, S. W., Giacherio, D. A., Blenk, N. & Kunkel, S. L. Endogenous norepinephrine regulates tumor necrosis factor-alpha production from macrophages *in vitro*. *J IMMUNOL* **152**, 3024–3031 (1994).
43. AI-ILQUIST, R. P. A study of the adrenotropic receptors. *AMER J PHYSIOL* **154**, 586 (1948).
44. Madden, K. S., Sanders, V. M. & Felten, D. L. Catecholamine influences and sympathetic neural modulation of immune responsiveness. *ANNU REV PHARMACOL* **35**, 417–448 (1995).
45. Sanders, V. M. & Straub, R. H. Norepinephrine, the β-Adrenergic receptor, and immunity. *BRAIN BEHAV IMMUN* **16**, 290–332, doi:10.1006/brbi.2001.0639 (2002).
46. Blais, V., Bounif, N. & Dubé, F. Characterization of a novel octopamine receptor expressed in the surf clam Spisula solidissima. *GEN COMP ENDOCR* **167**, 215 (2010).
47. Huang, J., Wu, S.-F., Li, X.-H., Adamo, S. A. & Ye, G.-Y. The characterization of a concentration-sensitive α-adrenergic-like octopamine receptor found on insect immune cells and its possible role in mediating stress hormone effects on immune function. *BRAIN BEHAV IMMUN* **26**, 942–950, doi:10.1016/j.bbi.2012.04.007 (2012).
48. McNeill, B. & Perry, S. F. Nitric oxide and the control of catecholamine secretion in rainbow trout *Oncorhynchus mykiss*. *J EXP BIOL* **208**, 2421–2431, doi:10.1242/jeb.01636 (2005).
49. Kim, D., Choi, H. J., Kim, S. W., Cho, S. W. & Hwang, O. Upregulation of catecholamine biosynthetic enzymes by nitric oxide. *J NEUROSCI RES* **72**, 98–104, doi:10.1002/jnr.10557 (2003).
50. Hughes Jr, T. K. *et al.* Interaction of immunoactive monokines (interleukin 1 and tumor necrosis factor) in the bivalve mollusc *Mytilus edulis*. *P NATL ACAD SCI USA* **87**, 4426–4429 (1990).
51. Kojima, H. *et al.* Fluorescent indicators for imaging nitric oxide production. *ANGEW CHEM INT EDIT* **38**, 3209–3212 (1999).

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Author contributions

Conceived and designed the experiments: L.S., L.W., Q.J. and Z.Z. Performed the experiments: Q.J., Z.Z., C.Y., J.W. and T.W. Analyzed the data: Q.J., Z.Z., L.W. and L.S. Contributed reagents/materials/analysis tools: Q.J., C.Y. and J.W. Wrote the manuscript: Q.J., Z.Z., L.S. and L.W.

Additional information

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