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 Ca²⁺ 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/Ca²⁺ regulatory pattern, and NO might have a feedback effect on the synthesis of norepinephrine in the scallop haemocytes.
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 vertebrates\(^3\). Catecholamines are synthesized from the catecholaminergic neuroendocrine system and released into serum during the immune stress\(^4\)–\(^8\). Catecholamines can be de novo synthesized in the immunocytes and secreted in an autocrine or a paracrine manner\(^8\). These released catecholamines can couple with the adreceptors (ARs) on the surface of immunocytes\(^3\), and can serve as an important immunomodulator in invertebrates\(^3\). For example, norepinephrine (NE), epinephrine and AR antagonist could negatively modulate the immune response against bacteria challenge in scallop *Chlamys farreri*\(^9\). NE could also regulate the level of reactive oxygen species (ROS) and phagocytosis of haemocytes via β-AR in oyster *Crassostrea gigas*\(^10\)–\(^12\), 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\(^13\).

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).

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 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 treated groups 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.
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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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, sGC activation⁴⁸,⁴⁹. However, the exact processes 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 Ca²⁺. 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.

**Methods**

Scallop haemocytes collection and primary haemocytes culture. Haemolymph and haemocytes were prepared as described previously by Hughes et al.⁴⁰ with some modifications. Haemolymph was aspirated by a syringe from the adductor muscle of scallop in ALS (Alseve) buffer (115.5 mmol L⁻¹ NaCl, 11.5 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 CaCl₂, 9.0 mmol L⁻¹.
Suppressed LPS-induced NO production by binding with SCIENTIFIC L-15, respectively. After incubated at 18°C, Sodium Nitroferricyanide (III) Dihydrate (SNP, Beyotime biotechnology, China) in MgSO₄, 41.0 mmol L⁻¹, 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 cAMP concentration 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 L⁻¹, respectively) (N = 6).

Measurement of Ca²⁺ level. Ca²⁺ levels in stimulation and control groups were examined by detecting the fluorescence of Fluo-3 AM (Beyotime biotechnology, China), the fluorescence probe of Ca²⁺. 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 withdrawn, the left cell pellets were washed with sterile PBS, and their fluorescence were detected by fluorescence spectrophotometer (HTACHL, Tokyo, Japan) with excitation wavelength of 488 nm and emission wavelength of 525 nm. Relative level of Ca²⁺ 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.

Figure 7 | Schema of signal pathways in present study. NE was secreted by autocrine or paracrine from C. farrelli haemocytes in response to LPS, and suppressed LPS-induced NO production by binding with α/β-AR and modulating the concentration of cAMP or Ca²⁺. NO could in turn promote the synthesis of NE.

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. 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 sterile PBS (136.89 mmol L⁻¹ NaCl, 2.68 mmol L⁻¹ KCl, 8.10 mmol L⁻¹ Na₂HPO₄, 1.47 mmol L⁻¹ KH₂PO₄, pH 7.4). The fluorescence was detected by fluorescence spectrophotometer (HTACHL, 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 (Abmova, USA). Briefly, NE was extracted from samples using a cia-diol-specific affinity gel, then acetylated 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).

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 adrenergic 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. 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 Ca²⁺. The trials were performed for sexptuples 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. 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 sterile PBS (136.89 mmol L⁻¹ NaCl, 2.68 mmol L⁻¹ KCl, 8.10 mmol L⁻¹ Na₂HPO₄, 1.47 mmol L⁻¹ KH₂PO₄, pH 7.4). The fluorescence was detected by fluorescence spectrophotometer (HTACHL, 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).

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.

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Author contributions
Conceived and designed the experiments: L.S., L.W., Q.J. and Z.Z. Performed the experiments: L.W., Z.Z., Q.J., Z.Z. and L.S. Analyzed the data: Q.J., Z.Z., L.W. and L.S. Contributed reagents/materials/analysis tools: Q.J., Z.Z., L.W. and L.S.

Additional information
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