Fish and Shellfish Immunology 93 (2019) 559–566

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of crustacean hyperglycemic hormone (CHH) on regulation of hemocyte intracellular signaling pathways and phagocytosis in white shrimp *Litopenaeus vannamei*

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ARTICLE INFO

Keywords:
Crustacean hyperglycemic hormone
Litopenaeus vannamei
Intracellular signaling pathway
Phagocytosis
Immune response

ABSTRACT

Shrimps like other arthropods rely on innate immune system, and may have some form of adaptive immunity in defending against pathogens. Phagocytosis is one of the oldest cellular processes, serving as a development process, a feeding mechanism and especially as a key defense reaction in innate immunity of all multicellular organisms. It is confirmed that crustacean hyperglycemic hormone (CHH) is one of the most important neuropeptides produced by Neuro-endocrine Immune (NEI) regulatory network, which undertakes important roles in various biological processes, especially in immune function and stress response. In this study, the recombinant *Litopenaeus vannamei* CHH (rLvCHH) was obtained from a bacterial expression system and the intracellular signaling pathways involved in the mechanism of phagocytosis after rLvCHH injection was investigated. The results showed that the contents of adenylyl cyclase (AC), phospholipase C (PLC) and calmodulin (CaM) in hemocytes were increased significantly after rLvCHH injection. Furthermore, the mRNA expression levels of NF-kB family members (relish and dorsal) and phagocytosis-related proteins in hemocytes were basically over-expressed after rLvCHH stimulation, while the expression level of NF-kB repressing factor (NKRF) gene was down-regulated significantly. Eventually, the total hemocyte count and phagocytic activity of hemocyte were dramatically enhanced within 3 h. Collectively, these results indicate that shrimps *L. vannamei* could carry out a simple but 'smart' NEI regulation through the action of neuroendocrine factors, which could couple with their receptors and trigger the downstream signaling pathways during the phagocytic responses of hemocytes.

1. Introduction

Phagocytosis is an evolutionary ancient defence mechanism shared by invertebrates and vertebrates, which play an important role in immune response. In the mammal immune system, macrophages and neutrophils are the 'specialized' phagocytes that internalize senescent and apoptotic cells, as well as invading pathogens [1]. Dendritic cells present processed antigens to lymphocytes, therefore linked innate and adaptive immunity [2,3]. The invertebrate immune system is based on effective humoral responses and various immunocyte (so-called hemocyte) processes such as reactive oxygen species production [4,5], antimicrobial peptide secretion [6–8], and encapsulation and phagocytosis [9–11]. Phagocytosis is one of the oldest cellular processes, serving as a development process, a feeding mechanism and especially as a key defense reaction in innate immunity of all multicellular organisms [1], which is considered a major line of nonspecific defense against pathogen invaders and foreign materials in invertebrates.

In general, phagocytosis is initiated by recognizing and binding of a target particle to the phagocytic cell, followed by uptake through cytoskeleton modification and intracellular vesicular transport to phagosomes where the engulfed target is destroyed. The latter stages of actin remodeling and vesicular trafficking are probably conserved between invertebrates and higher animals. Briefly, the process of phagocytosis involves recognition, adhesion, aggregation, ingestion and clearance [12,13]. Furthermore, immune system as an important part of neuroendocrine-immune (NEI) regulatory network, together with nervous system and endocrine system, form a bidirectional regulation to maintain homeostasis with the involvement of neuroendocrine factors [14–16]. Many studies are only dominated on exploration of invertebrate immunity, with little attention to the effect of neuroendocrine factors on immune mechanism. Thus, identifying the key neuroendocrine factor from NEI regulatory network in invertebrate phyla

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https://doi.org/10.1016/j.fsi.2019.07.051
Received 13 February 2019; Received in revised form 13 July 2019; Accepted 17 July 2019

and how it influences the immune response mechanism remains a critical priority.

In crustaceans, X-organ/sinus gland complex (XO/SG complex) of the eyestalks is a fundamental neuroendocrine organ, secreting a variety of neuropeptides to regulate physiological activities [17,18]. The most abundant neuropeptide produced by this complex is crustacean hyperglycemic hormone (CHH), which is involved in glucose regulation and other various biological processes, especially in immune function and stress response [19,20]. After exposure to environmental stress such as hypoxia, salinity, thermal stress, emersion or heavy metal pollutants, a drastic increase in CHH level has been observed [21–23]. Similarly, exposure to viral diseases [24] and parasitic dinoflagellates [25], is also associated with rapid increase in CHH level. Moreover, it is well known that environmental parameters such as temperature, salinity and dissolved oxygen can affect the immune response of crustaceans [26–28]. Likewise, previous studies showed that disease outbreaks could also suppress the immune system and cause oxidative damage to organism [29]. Thus, the role of CHH can be regarded as a stress hormone center of immune responses in the event of environment changes or disease outbreaks. Moreover, the corticotropin-releasing hormone (CRH), adrenocorticotropin hormone (ACTH), of the biogenic amine axis is the main origin of the stress response in vertebrates and invertebrates (molluscs) [30]. In invertebrates, the process is simplified, for rather than several organs, such as the hypothalamus, pituitary, and adrenal glands, being involved, the response is concentrated in phagocytic haemocytes, which harbors all the relevant molecules. The release of biogenic amine is believed to be a proto-stress response [30].

Hormones or neurotransmitters accomplish their immune functions by binding to their specific receptors on the surface of immunocyte, which involves the activation of extracellular signaling moleculars. Hemocytes in invertebrates are regarded as main immune cells. On the one hand, previous results have revealed that membrane-bound guanylyl cyclase (mGC) plays the role of CHH receptor for the actions of CHH on hemocytes and that CHH activates the cyclic guanosine monophosphate (cGMP) pathway through activation of mGC activity [31]. And the active cGMP could elevate the protein kinases G (PKG) level in order to regulate various physiological activities [32]. On the other hand, it is reported that the biogenic amines receptors are G protein-coupled receptors (GPCR), which could activate downstream signaling molecules and produce cellular signaling effects [33,34]. Chang et al. showed that dopamine (DA) might modulate the levels of immune parameters such as respiratory bursts and superoxide dismutase activities mainly through type 1 DA receptors in *Macrobrachium rosenbergii* [34]. Crustacean hemocytes play important roles in the host immune responses, such as recognition, phagocytosis, and encapsulation [35,36]. Among them, phagocytosis is the core of crustacean immune defense, which has been pointed out that circulating phagocytes (hemocytes) are the ancient and conserved link between immune and neuroendocrine functions [37]. A number of researches showed that POMC-derived fragments (ACTH/α-MSH, β-MSH, γ –MSH and opioid β-endorphin), together with biogenic amines participate in phagocytosis [38–40]. It is demonstrated that in vivo injection of recombinant CHH significantly increases pathogen clearance ability [41], while the phagocytic activity and signal transduction mechanism of phagocytosis in crustaceans remain unclear in *Litopenaeus vannamei (L. vannamei)*.

Shrimp aquaculture accounts for a very important part in the economics of the world. Most part of the shrimp aquaculture production came from *L. vannamei* which accounts for 80% of the whole shrimp production. At present, the shrimp farming industry is threatened by various diseases related to environment changes and some farming activities, which causes serious economic losses [42]. Therefore, investigations of the immunity of shrimp must be very helpful to healthy aquaculture of crustaceans. This study aimed at revealing the role of the recombinant *L. vannamei* CHH in immune response, creatively, detecting the concentration of intracellular signaling pathway factors and gene expression of NF-kB family members, and phagocytosis-related proteins in hemocytes, conclusively, assay phagocytosis-related immune response parameters. Thereby, this research will provide a theoretical basis for understanding the immune signaling pathways and valuable evidence for exploring the phagocytosis mechanism in the shrimp under injection of rLvCHH.

2. Materials and methods

All the experiments were carried out in accordance with the Animal Care and Use Committee of Ocean University of China, following the guidelines of animal experiments of Ocean University of China, under permit No. SCXY-S20160812.

2.1. Animals preparation

Subadult *L. vannamei*, averaging 11.0 ± 0.8 cm in body length and 12.5 ± 1.6 g in body weight, were obtained from a commercial farm in Shazikou, Qingdao, China. The shrimp were acclimated in indoor tanks (40 cm × 50 cm × 60 cm) containing aerated natural seawater (salinity 31‰, pH 8.2) with an air-lift at 25 ± 0.5 °C for two weeks prior to experiments. During the acclimation period, one third to half of the water in each tank was replaced twice daily and the shrimp were fed with a formulated commercial diet daily (Haiyue Company, Qingdao). Apparently healthy shrimp (physical integrity without injury, normal color and good viability) at the intermolt stage were chosen for the following experiment. The intermolt phase of shrimp was determined following the criteria of Robertson et al. (1987) [43].

2.2. Preparation of recombinant crustacean hyperglycemic hormone in L. vannamei

The rLvCHH (GenBank: HM748790.2) in this study with an expression vector pET-32a (+) (TaKaRa, Dalian, China) was used to transform *Escherichia coli BL21* (DE3) (TransGen Biotech, Beijing, China). The expression and identification of rLvCHH was carried out on modifying the expression method of Liu et al. (2014) [44] and designed on the base of expression method of Zhang et al. (2018) [45]. (Specific primers LvCHHBamH I: CGGGATCCATGACTGCCTTCCGT and LvCHHXho I: CCGCTCGAGTTTCCCGACCATCTG). The efficiency and specificity of polyclonal antibodies against rLvCHH were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

2.3. Injection of recombinant LvCHH and gain-of-function analysis of rLvCHH in vivo

504 Shrimps (*L. vannamei*) were randomly and equally assigned to each experimental or control group, each group included 168 shrimps and was set 3 replicates. Experimental groups were injected in the abdominal muscle with 0.2 μg or 2 μg of rLvCHH protein dissolved in shrimp saline solution, while the control group received the same volume of saline solution (NaCl 0.40 mol/L, $CaCl_2{\cdot}2H_2O$ 0.01 mol/L, $Na_2HPO_4{\cdot}12H_2O$ 0.0004 mol/L, KCl 0.009 mol/L, $MgCl_2{\cdot}6H_2O$ 0.02 mol/L, Tris 0.035 mol/L, pH 7.45, osmolality 780 mOsm/kg) (Solarbio, Beijing, China). The injected dose were based on Laura et al. (2017) [46] and Wang et al. (2017) [47]. The experimental conditions were identical to those of the acclimation period and there was no death during the experiment time. Eight shrimps were randomly sampled from each replicate at 0, 1, 3, 6, 12, 24 and 48 h, then hemolymph samples of eight individual shrimp were withdrawn from the ventral sinus at the third segment of walking leg using a 1-ml syringe with a 25 G-needle containing cooled anticoagulant (450 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L EDTA-Na_2 and 10 mmol/L HEPES, pH 7.45, 780 mOsm/kg) (Solarbio, Beijing, China) in a 1:1 ratio [48]. Samples of the hemolymph from each replicate were gently mixed in RNase-free tubes for physiological parameters analysis as follows.

2.4. Preparation of plasma and hemocytes

After collection, samples were immediately centrifuged in a refrigerated centrifuge (4 °C) for 10 min at 700 g. Following centrifugation, and the supernatant (plasma) was stored −80 °C. The pellet was suspended in 1 mL Trizol reagent (TransGen, China) and stored at −80 °C until for total RNA extraction. Hemocyte lysate supernatant (HLS) was prepared using the modified method of Smith and Söderhäll (1991) [49]. The resultant cell pellet was rinsed, re-suspended gently in 1.0 mL of sterilizing shrimp normal saline. And then the cells were broken into pieces for 1 min by Ultrasonic Cell Disruption System with output power at 20 W and duty cycle at 30% in the ice-bath and then centrifuged at 15000 g for 20 min at 4 °C. The resultant fluid (HLS) was stored at −80 °C for measurements of AC, PLC and CaM concentration.

2.5. Intracellular signaling transduction factor assay

The concentration of adenylyl cyclase (AC), phospholipase C (PLC) and Calmodulin (CaM) in HLS were measured using shrimp AC ELISA kit ((BP-E94050, Shanghai Lengton Bioscience Co., LTD, China), shrimp PLC ELISA kit (BP-E94032, Shanghai Lengton Bioscience Co., LTD, China) and shrimp CaM ELISA kit (BP-E94030, Shanghai Lengton Bioscience Co., LTD, China). All of them were measured by ELISA kit according to the manufacturer's instructions.

2.6. RNA isolation, cDNA synthesis and qRT-PCR

Total RNA from hemocytes in the shrimp was isolated using RNAiso Plus (TaKaRa, Dalian, China). The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm (A260/A280) using NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was monitored on 1% agarose gels, according to the manufacturer's instructions.

The expressions of genes were detected by real-time quantitative PCR (qPCR), and the primers are shown in Table 1. Two commonly used reference genes,18S and β-actin, were tested to evaluate their stability as endogenous control gene using the BestKeeper method described by Pfaffl et al. (2004) [50]. β-actin was found to have the lowest variation during the experiment and was then chosen as the housekeeping gene for the rest of the analyses. For all standard curves, the primer amplification efficiencies of genes were 96.3–99.5% and $0.971 < R^2 < 0.997$ respectively. The expression levels were compared with the relative Ct method [51].

2.7. Immune response parameters assay

After hemolymph was collected, 50 μL hemocyte suspension was pipetted immediately into a new tube containing an equal volume of 10% formaldehyde for 30 min at 4 °C 20 μL hemocyte fixed by formaldehyde dropwise was added into the hemocytometer, and then it was transferred into the optical microscope (Olympus, Japan).

Table 1
Primer sequences and function used in this study.

Primer name	Primer sequence (5′–3′)	Genbank number
Relish-F	AATATTGTTGGTGGCCTGGGT	*SRP132193*
Relish-R	TCCACTTGGCTTTGAGGGTT	*SRP132193*
Dorsal-F	TTGCGACCACCAGACAAGAG	*SRP132193*
Dorsal-R	GCAAGGTAACGACTAATCTTCTCTG	*SRP132193*
NKRF-F	AGTGACAACCAGACGGCAA	*No.* KY864366
NKRF-R	CTGTATTGCTGGTCTGTTTCG	*No.* KY864366
Peroxinectin-F	AACCTGGCTTGACTGCTATT	KC708021.1
Peroxinectin-R	CTGCCACCACAAACCTTCTA	KC708021.1
Mas-like protein-F	CAGTCGTTTGACCGCATTT	JX644451.1
Mas-like protein-R	GCGATGATTCCAAGCCTCT	JX644451.1
Dynamin-F	TGGTACTAAGTCCCGTGTTGTCT	*SRR346404*
Dynamin-R	ATTCCTCCGAGCTGGTGTAT	*SRR346404*
β-actin-F	AAAGCTCCGTCGTCAGTA	AF300705
β-actin-R	AAGCCTTCGTCTCCAAAT	AF300705

Phagocytic activity of hemocyte was measured using *Vibrio alginolyticus* by the method of Yue et al. (2010) [52]. Briefly, hemocytes were separated from 200 μL hemolymph and washed with sterilizing shrimp normal saline by centrifugation at 700g and 4 °C, volume of 100 μL of hemocytes suspension and equal volume of bacterial suspension (1×10^7 cfu mL^{-1}) were placed into a plastic micro plate. The mixture was incubated in a moist chamber at 25 °C for 30 min. After that, a drop of the mixture was pipetted onto a glass slide and then dried at room temperature (25 °C), fixed in methanol, stained with Giemsa stain (Solarbio, Beijing, China), decolorized in MilliQ water, air-dried and observed using Olympus light microscope (10 × ocular, 100 × oil immersion objective). The number of phagocytic hemocytes among random 200 hemocytes was counted. Phagocytic activity, defined as phagocytic percent was calculated as: phagocytic percent (%) = (number of phagocytic hemocytes/200 hemocytes) × 100%.

2.8. Statistical analysis

All data are presented as mean ± standard error. Statistical analyses were performed by one-way analysis of variance (ANOVA) using the software SPSS 25.0 (SPSS Inc., Chicago, IL, USA) and Duncan Multiple Range test was used to identify significant differences among different treatments at the same time point. The level of statistical significance was set at $P < 0.05$.

3. Result

3.1. Effect of rLvCHH on signaling pathway factors concentration in hemocytes

The concentration of AC, PLC and CaM (Fig. 1) in supernatants of hemocytes after rLvCHH injection was quantified to evaluate the downstream signaling pathway triggered by high level of CHH and DA. The results showed that the AC and PLC concentrations presented an uptrend in relation to the control group under rLvCHH stimulation, and reached the summit at 6 h and 12 h respectively ($P < 0.05$) (Fig. 1A and B). In addition, the CaM concentration was significantly elevated in rLvCHH injection groups (0.2 and 2 μg/shrimp) ($P < 0.05$) comparing with that in control group and reached the maximum at 12 h (Fig. 1C). Then both of them restored to the basic level at 48 h with no significant difference in comparison with that in the control group ($P > 0.05$).

3.2. Effect of rLvCHH on mRNA expression of NF-kB family memebers in hemocytes

After shrimps injected with 0.2 and 2 μg of rLvCHH, the mRNA expression levels of relish and dorsal were overexpressed remarkably ($P < 0.05$) and reached highest values at 3 h dose-dependently ($P < 0.05$) (Fig. 2A and B). Then there was a significant decrease in the mRNA levels of relish and dorsal, especially in relish, while the significant overexpression of relish gene was observed again at 24 h ($P < 0.05$). Conversely, the expression level of NF-kB repressing factor (NKRF) gene was down-regulated significantly and reached the lowest level at 6 h after rLvCHH stimulation ($P < 0.05$) (Fig. 2C).

3.3. Effect of rLvCHH on mRNA expression of phagocytosis-associated proteins in hemocytes

The transcription alternations of phagocytosis-associated proteins, including peroxinectin, masquerade (mas)-like protein and dynamin were detected to investigate the potential mechanism of phagocytosis in immunoregulation induced by rLvCHH. As shown in Fig. 3, the mRNA expression of the three examined genes were basically upregulated

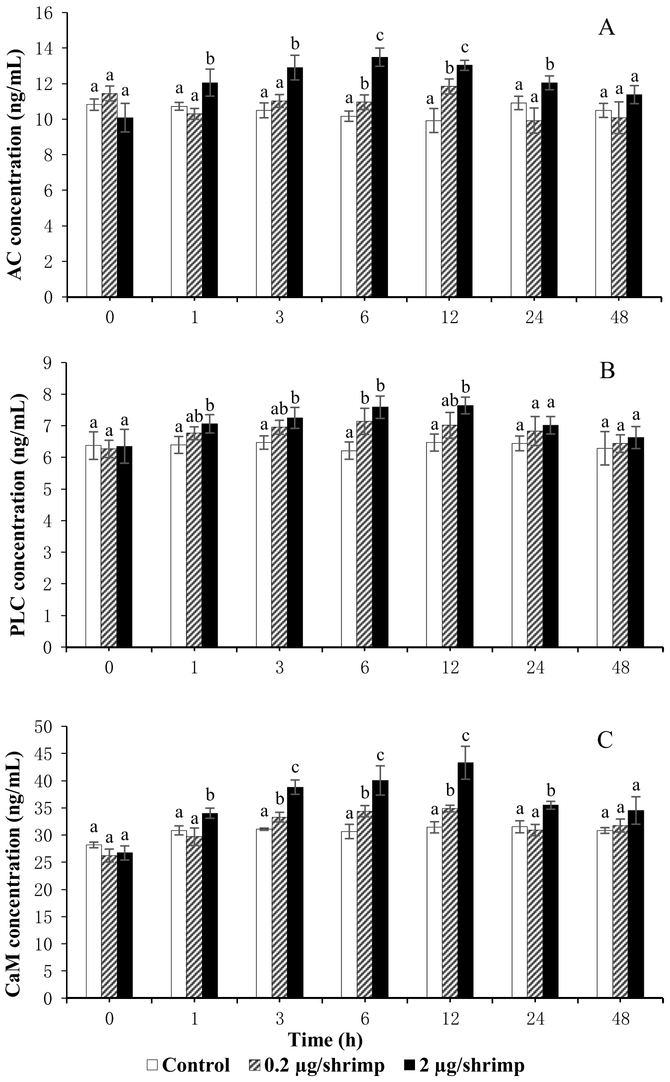

Fig. 1. Second messenger synthetase and Calmodulin concentrations in hemolymph of *L. vannamei* under low (0.2 μg/shrimp) and high (2 μg/shrimp) concentrations of rLvCHH injection (A: AC; B: PLC; C: CaM). Data are means + SEM. (n = 8). Bars with different letters represent significant differences ($P < 0.05$) among the control and treated groups.

during the early stages after rLvCHH stimulation and reached the maximum at 3 h ($P < 0.05$), while no obvious changes were observed in the control group (Fig. 3A, B, C). Subsequently, an obvious decline was observed in the gene expression level of these proteins. Moreover, there was a significant difference between the low (0.2 μg/shrimp) and high (2 μg/shrimp) rLvCHH injection groups in peak values ($P < 0.05$).

3.4. Effect of rLvCHH on immune response parameters

To further confirm the possible mediation of rLvCHH to phagocytosis, total hemocyte count (THC) and phagocytic activities after rLvCHH administration. As presented in Fig. 4, after rLvCHH stimulation, total hemocyte count (THC) and phagocytic activities were dramatically increased ($P < 0.05$) in comparison with the control group and peaked at 3 h dose-dependently (Fig. 4A and B), both of them subsequently restored to the basic level at 24 h.

4. Discussion

The communication and interaction between neuroendocrine and

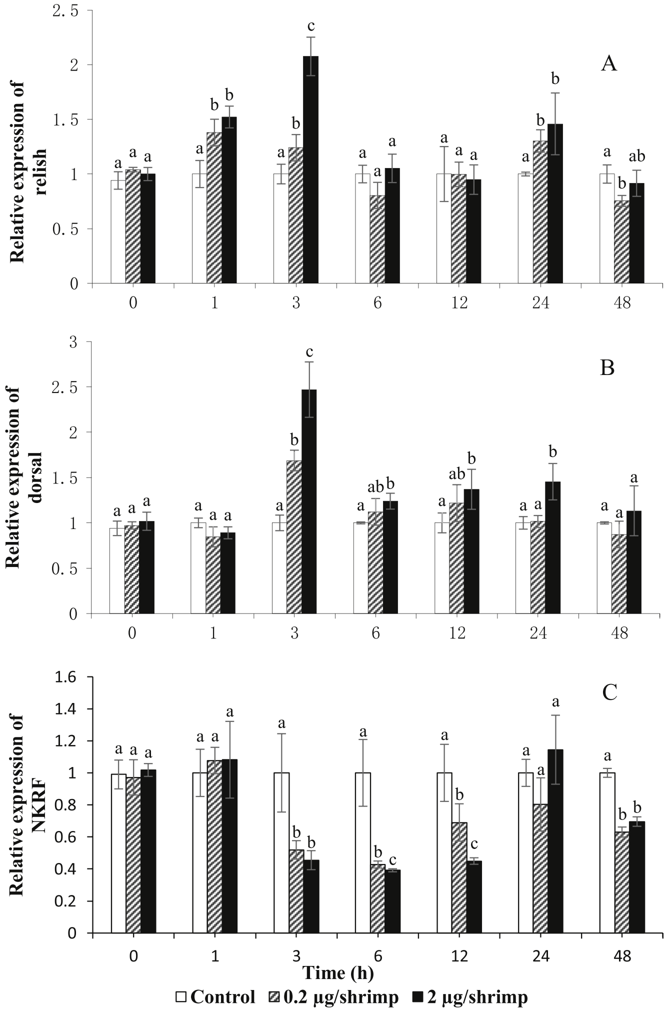

Fig. 2. The mRNA expression of NF-kB family members in hemocytes of *L. vannamei* under low (0.2 μg/shrimp) and high (2 μg/shrimp) concentrations of rLvCHH injection (A: relish; B: dorsal; C: NKRF). Data are means + SEM. (n = 8). Bars with different letters represent significant differences ($P < 0.05$) among the control and treated groups.

immune system is an important regulatory mechanism for the maintenance of immune homeostasis in vertebrates, and it also plays an important role in the immunomodulation of some invertebrates [53,54]. But the signaling pathways of immune response in invertebrates and their differences with vertebrates are still far from well-understood. The present study focused on the immunoregulation of CHH to further explore the underlying signaling pathways mediated by circulating hemocytes of white shrimp *L. vannamei* after rLvCHH stimulation, in order to explore the mechanism of phagocytosis regulated by neuroendocrine factors and enrich our knowledge of NEI network in marine crustaceans.

Neuroendocrine factors, including neuroendocrine hormones and neurotransmitters, are of great significance for the integrity of NEI network and the maintenance of homeostasis. CHH is one of the most important neuroendocrine hormones and undertake comprehensive physiological functions, especially in immune function and stress response, which has attracted much attention. Lorenzon et al. (1997) have shown that lipopolysaccharides (LPS) induce hyperglycemia, which show no response in eyestalkless animals [55], and LPS challenge was later to be shown to induce CHH release [23]. Recently, wang et al. (2017) reported that recombinant LvCHHs in *E.coli* might contribute to the robust immune response of host by powering the hemocytes as well as activating the expression of immune-related genes directly [47].

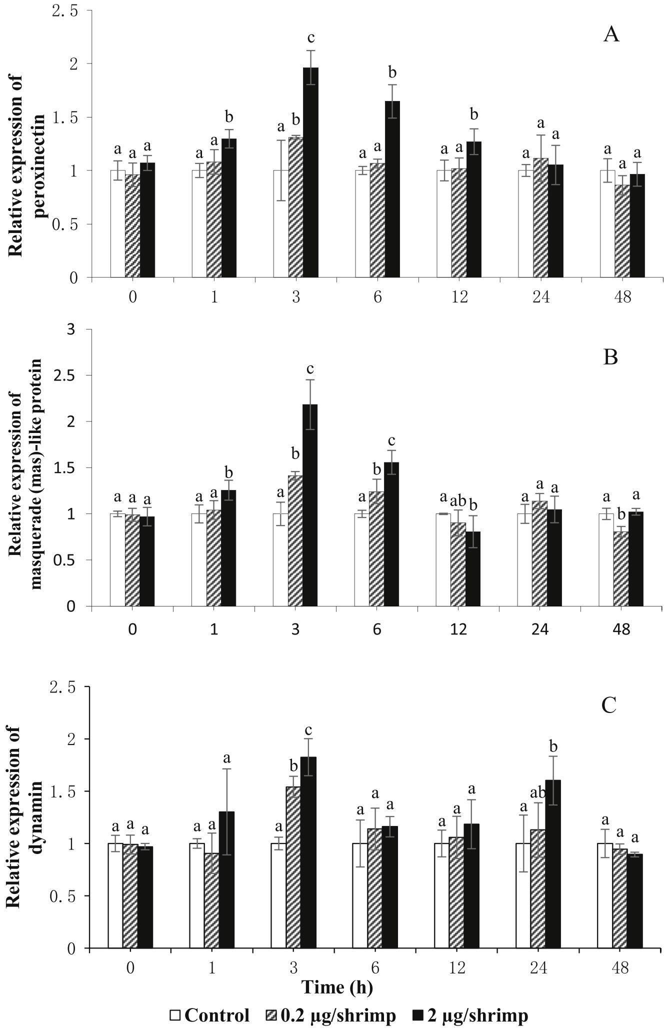

Fig. 3. Phagocytosis -related proteins mRNA expression in hemocytes of *L. vannamei* under low (0.2 μg/shrimp) and high (2 μg/shrimp) concentrations of rLvCHH injection (A: peroxinectin; B: mas-like protein; C: dynamin). Data are means + SEM. (n = 8). Bars with different letters represent significant differences ($P < 0.05$) among the control and treated groups.

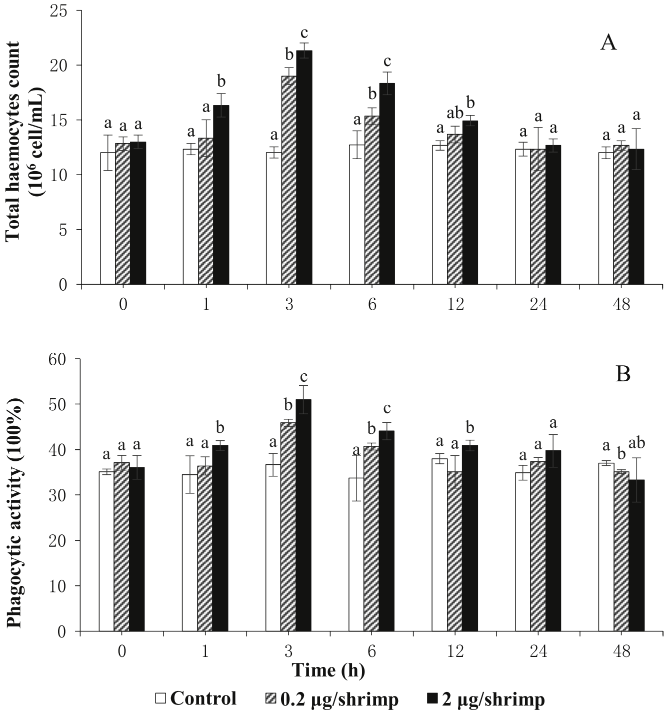

Fig. 4. THC and phagocytic activities of *L. vannamei* under low (0.2 μg/shrimp) and high (2 μg/shrimp) concentrations of rLvCHH injection (A: THC; B: phagocytic activity). Data are means + SEM. (n = 8). Bars with different letters represent significant differences ($P < 0.05$) among the control and treated groups.

Furthermore, when threatened by extrinsic stressors, CHH can be regarded as a stress hormone center of immune responses in crustaceans. Blue crabs (*Callinectes sapidus*) exposed to hypoxic conditions responded with concomitant release of both CHH from eyestalk and pericardial organ [56]. In the present study, we expressed LvCHH from eyestalk of *L. vannamei* in *E. coli* and characterized the hyperglycemic activity of the recombinant protein in *L. vannamei.* Obtained data have demonstrated the ability to elevate the glucose level in hemolymph of rLvCHH by intramuscular injection [57], which suggested that rLvCHH could function as CHH in *L. vannamei* and might play pivotal roles in immune regulation. Furthermore, it is interesting that the concentrations of DA in hemolymph changed temporally after rLvCHH stimulation compared with those in the control group, which presented a uptrend from the whole experimental period and reached the maximum value at 12 h [57]. Previous reports have revealed the immune response mechanism of DA under environmental stress in crustacean [58–60]. In invertebrates, it has been revealed that environmental stress could induce the rapid release of CHH and DA accompanied by hyperglycemia response in intact individuals. And previous researches have reported that DA seems to have an effect on the modulation of CHH level. For example, the results of Camacho-Jiménez et al. (2017) suggested that DA could stimulate the secretion of CHH from the XO–SG complex in the eyestalk of *L. vannamei* [61], which was in agreement with those obtained in another shrimp, *P. monodon* [62], the crab *Oziothelphusa senex senex* [63], and the freshwater prawn *Macrobrachium malcolmsonii* [64], where DA was shown to elevate hemolymph glucose in intact organisms. Based on these results, the fluctuation of DA after rLvCHH injection might be the result of the feedback effect of a sharp rise in glucose levels, while this effect was relieved subsequently with the decrease of CHH concentration in order to maintain immune homeostasis of *L. vannamei*. However, some studies showed that DA has no significant effect on the release of CHH and on the increase of hemolymph glucose level in *Palaemon elegans* [65], which are contrasting with the above researches. Collectively, the role of DA in the release of CHH seems to be controversial, currently, different results could be due to differences in species or experimental design. However, according to our previous researches, it is concluded that the stimulation of CHH influenced the level of DA, although there were nearly no reports on this issue and the exact mechanism needs to be further explored. In fact, NEI regulatory network is far more complex than well-understood, and our researches might be the first step to investigate the effect of CHH injection in vivo on DA variation.

Neurohormones and neurotransmitters are able to bind specific immunocyte receptors, thus modulating their activities through signal pathways and triggering the events inside the cell. Previous results have revealed that a membrane-bound GC could act as the CHH receptor and CHH could activate cellular signaling transduction mechanisms by directly activating mGC without binding to G-proteins in the Y-organ and hemocyte. Data concurrent with the suggestion are those showing that CHH increased in a dose-dependent manner the membrane-bound GC activity of hemocytes and Y-organ, which leads to an increase in protein kinase PKG through activation of cGMP activity [31,32,66,67]. In our previous study, the mRNA expression level of mGC in hemocytes was examined and changed consistently with the variation of PKG after rLvCHH stimulation, which confirm the role of mGC acting as the primary signal processor in CHH-mediated immunoregulation pathways

[57]. Furthermore, it is worth mentioning that DA receptors are an evolutionarily conserved family of G protein coupled receptors (GPCRs). In mammals, the DA receptors are broadly classified into two subfamilies: type 1 DA receptors (D1/D1A, D1B/D5, D1C, D1D) and type 2 DA receptors (D2, D3, D4) [33]. In crustacean, little research has been done on DA receptors. There are only four well-characterized DA receptors in the phylum arthropoda, three types DA receptors from the spiny lobster, *Panulirus interruptus*: D1αPan, D1βPan and D2αPan [68,69]. One type from the *Penaeus monodon*: PemDopR1, which has the highest similarity to D1β of the spiny lobster [70]. D1αPan could couple with Gs to increase cAMP, while D1βPan couples with Gs and Gz to produce a net increase in cAMP. As for D2αPan, this receptor positively couples with cAMP through multiple Gi/o proteins via two discrete pathways: one is a Gα mediated inhibition of adenylyl cyclase (AC), leading to a decrease in cAMP, the other is a Gβγ-mediated activation of phospholipase C (PLC), leading to an increase in cAMP. Moreover, Rashid et al. (2010) reported a novel D1-D2 DA receptor complex identified in rat brain, which could activate Gq/11 and PLC and induce an intracellular calcium release and a rapid phosphorylation of CaMKII in the nucleus accumbens [71]. The data presented in our previous study showd that the mRNA expression levels of DA2R was substantially decreased at 6 h and minimized at 12 h [57]. On the contrary, a significant increase in the concentration of AC, PLC and CaM in haemocyte after rLvCHH stimulation was observed in the present study, it is discernable that the fluctuation of PLC and CaM showed a similar trend, which all reached the summit at 12 h. Recently, the mRNA expression level of Gi and Gq was also identified by our groups respectively [57], which presented a downtrend and an uptrend respectively after rLvCHH injection, these trends might be closely associated with the variation of DA2R transcripts and the action of AC, PLC and CaM. Furthermore, Zhang et al. (2018) have revealed that DA seems to transduce the signals by cAMP-, CaM-pathways in order to regulate immune response of *L. vannamei*, including phagocytosis of hemocytes [45]. To sum up the above research results, the change of PLC and CaM might be the result of DA receptor regulation. Furthermore, the concentration of AC showed a drastic variation after rLvCHH stimulation, suggesting that other types of biogenic amine receptors probably also exist in hemocytes of *L. vannamei* to regulate the immune response and further researches are needed to clarify their role in signaling pathways.

Hemocytes, functionally similar to mammalian macrophages, carried out the cellular response and primarily acted to endocytose, phagocytose or encapsulate foreign material. Particularly, phagocytosis played a major part in limiting infection in invertebrates [35,36,72,73]. It is worth mentioning that a higher-than-normal number of circulating hemocytes in crustaceans correlate with an increased resistance to pathogens [74], so it can be considered as an indicator of shrimp immunity ability [75]. In our experimental conditions, a significant increase of THC was observed in *L. vannamei* after rLvCHH injection, and the phagocytic activity increased significantly in a dose-dependent manner of rLvCHH injection. Additionally, the study by Speck-Lascola (2007) suggested that PKG is essential for phagocytosis and production of LPS induced pro-inflammatory molecules in macrophages [76], but little is known in aquatic animals. In our previous study, there is a significant increase of the contents of PKG and PKC in hemocytes. Particularly, PKG contents was increased to a significant level at the early stages post-rLvCHH injection and reached the summit at 3 h, but the content of PKC increased at late stages [57]. Furthermore, the gene expression level of relish and dorsal, increased remarkably and reached the peaks at 3 h after rLvCHH stimulation, which are consistent with the increase of THC and the phagocytic activity, while the expression level of NKRF mRNA transcript was significantly lower than the control group. The NF-kB family is a group of conserved and structurally related transcriptional factors involving in various key biological processes in both vertebrates and invertebrates [77,78]. Dorsal and Relish are the important two members of the NF-kB family in crustaceans, governing a broad spectrum of cellular responses in immunity [79,80]. Previous reports have demonstrated that the mRNA expression of relish and dorsal were significantly increased when stimulated by *Vibrio anguillarium*, and it was found that the expression of antimicrobial peptide Penaeidin 5 was significantly reduced when dorsal gene was silenced [81,82]. Conclusively, although relish and dorsal might play different roles in shrimp immunity, as was shown in this study, the two members of NF-kB family seem to play an important role in the action of phagocytosis of hemocytes, which might be mediated by the activation of PKG. Interestingly, the mRNA expression level of NKRF decreased dramatically post-rLvCHH injection. In mammals, NKRF was firstly identified as a NF-kB interacting protein with an inhibitory effect on NF-kB-activated promoters, and was then classified into a class of active repressors that act by direct protein-protein interaction [83]. The current study revealed that the role of NKRF in the immune regulation and the interaction with relish and dorsal seems to be similar to the reports in vertebrates. However, Qiu et al. (2017) have reported that LvNKRF did not show inhibitory but instead excitatory effects on activities of shrimp NF-kB [84], which was contrary to those of mammalian NKRFs. In fact, currently, NKRFs have hardly been functionally studied outside mammals. The knowledge mainly from mammals is limited for exploring the evolutionary mechanisms of NKRFs. It is speculated that the different roles of NKRFs in the regulation of immune response may be due to different experimental conditions and design. This study aimed to seek the crucial signaling pathway factors in the activation of phagocytic activity, thus, there's little doubt that more evidence should be provided to further elucidate the roles of NKRFs in the NF-kB pathway during evolution. Clearly, it would be interesting to verify which signaling pathways involved in phagocytosis by haemocytes is the most important when shrimp are challenged with rLvCHH.

Phagocytosis has been extensively studied in mammalian macrophages [1], but poorly studied in decapod crustaceans. Bayne (1990) reported that the recognition of the target was either directed with receptor (lectins) binding to the target surface or mediated via opsonization factors that marked the particles for phagocytosis [85]. As was reported in previous studies, integrins, which played crucial roles in phagocytosis [86–88], resembled the “masters and slaves” in phagocytic transport [87]. Both peroxinectin and masquerade (mas)-like protein were isolated from the hemocytes of crayfish *P. leniusculus* firstly and acted as cell adhesion factors and opsonins [89–91]. Peroxinectin, widespread in invertebrates, was released by exocytosis from the hemocytes, which played an important role as an immune protein in defending against pathogens [92]. Mas-like protein had dual functions, as an LPS and β-1,3-glucan recognition protein and as a cell adhesion and/or opsonic protein, which could greatly accelerate the rate of phagocytosis in crayfish *Pacifastacus leniusculus* [93]. In the present study, to demonstrate the phagocytic mechanism of *L. vannamei*, the mRNA expression levels of peroxinectin and mas-like protein were investigated, the results of which indicated that the two proteins mRNA level were significantly up-regulated after rLvCHH injection and reached the peak values at 3 h, and all the parameters gradually recovered to the control levels at 24 h, which had a close time correlation with the changes in the phagocytic activity. Moreover, phagocytosis was an actin-dependent process which represented an essential branch of the immune system. Dynamin was essential for the formation of clathrin-coated vesicle in endocytosis, in transport from the trans-Golgi network, as well as for ligand uptake through caveolae [94–97]. It was confirmed in this study that this molecule was involved in the regulation of phagocytosis in shrimp, among which the gene expression level of dynamin was significantly up-regulated and reached the highest level at 3 h. Furthermore, the integrin mRNA level was also up-regulated significantly in our previous study [57]. Thus, taking the results of previous studies and our own into combined consideration, we suggested that after injection of rLvCHH, the three cell adhesion factors, namely, integrin, peroxinectin and mas-like protein were involved in the recognition, adhesion and aggregation processes of phagocytosis.

Briefly, these three proteins participated in phagocytic cup formation. Subsequently, dynamin interacted with other endocytic proteins, and participated in invagination and fission to form phagosomes, and eventually, the phagosomes were destroyed by lysosome or oxidation sterilization. However, interestingly, a significant decrease was observed in the transcripts level of these four phagocytosis -related proteins and the expression level of relish and dorsal starting from 6 h after injection of rLvCHH compared with before, which are contrary to the variation of DA contents and the action of CaM and PKC [57]. Plows et al. (2004) identified that the PKC pathway was the key regulator of the phagocytic response in *Lymnaea stagnalis* [98]. Likewise, Humphries and Yoshino (2003) suggested that PKC may be involved in phagocytosis of *Biomphalaria glabrata* haemocytes [99]. Collectively, a possible explanation for this fluctuation might be that DA was induced by injection of rLvCHH at the late stages of the immune response in order to avoid the damage caused by excessive inflammation, restituting the homeostasis [57].

5. Conclusion

The findings of this investigation suggested that rLvCHH could significantly enhance the phagocytic responses in *L. vannamei* through the activation of mGC and DA receptors. On the one hand, the higher level of CHH might trigger PKG pathway via the action of mGC. On the other hand, the production of DA after rLvCHH simulation might influence the activities of CaM/PKC pathway by activating their corresponding receptors. Collectively, the above two signaling pathways might activate phagocytosis-related proteins and the phagocytic activity through the activation of NF-kB pathway to regulate immune responses and maintain body health. Shrimps possess only primitive nervous system and immune-related tissues. They do not have a complicated nervous system or endocrine and immune organs to conduct elaborate physiological regulation. Thus, the researches on the mechanism of immune homeostasis in shrimps remain difficult and scarce. The results presented in this study will contribute to clarify the elaborate potential process of phagocytosis in shrimps, *L. vannamei* and enrich some evidence for existence of a simple but ‘smart’ NEI network in shrimps.

Acknowledgments

The work was supported by State Oceanic Administration Specific Public Project of China (201305005). The authors are thankful to the staff at the Laboratory of Environmental Physiology of Aquatic Animal for their help with sampling and taking care of the shrimps.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.051.

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