Crustacean hyperglycemic hormones directly modulate the immune
response of hemocytes in shrimp *Litopenaeus vannamei*

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Abstract

A robust immune response against invading pathogens is crucial for host to survive, which depends greatly on the well balance of metabolism. Increasing evidence has indicated that some metabolic hormones, such as insulin, could modulate immune responses directly. Crustacean hyperglycemic hormone (CHH) family is a group of ecdysozoans-specific peptide hormone involved in glucose metabolism and other biological events. In the present study, two members of CHH family (designated as LvCHH I and LvCHH II) in shrimp *Litopenaeus vannamei* with one and two crustacean neurohormone domains respectively were chosen to investigate their putative modulatory roles in both glucose metabolism and immune response. LvCHH

I and LvCHH II were both expressed in the sinus gland and lamina ganglionalis of eyestalks and were significantly induced after white spot syndrome virus (WSSV) infection. Meanwhile, significant increases of hemolymph glucose levels were observed in shrimp at 12 and 24 h after WSSV infection while the glucose inside the hemocytes decreased at 6 h and then increased at 12 h. Gain-of-function of rLvCHHs was subsequently conducted *in vivo* by injecting the recombinant proteins (rLvCHH I and rLvCHH II). The hemolymph glucose increased significantly from 0.5 h to 3 h after the shrimps received an injection of rLvCHH I, while it decreased at 0.5 h and increased afterward at 3 h post rLvCHH II injection. At the meantime, significant decreases of reactive oxygen species level in hemocytes were observed at 3 h and 6 h post rLvCHH I injection, while it remained unchanged in rLvCHH II injection group. rLvCHH I and rLvCHH II could bind to the cytomembrane of primary shrimp hemocytes *in vitro*, and the expressions of superoxide dismutase and LvRelish increased when the hemocytes were incubated with rLvCHH I for 3 hours. Meanwhile, the expression of antimicrobial peptides, crustin and penaeidin-4, were also induced by rLvCHH I and rLvCHH II. These results demonstrated that host immune response, in addition to glucose metabolism, could be directly modulated by LvCHH family, and the present study provided new insights into the immunomodulation role of metabolic hormones in invertebrate.

Key words: Shrimp; WSSV infection; Crustacean hyperglycemic hormones; Glucose metabolism; Immunomodulation

1. Introduction

The multicellular organisms depend greatly on their robust immune system to survive through the invading pathogens [1]. Immune responses of host could be rapidly induced after infection to eliminate those pathogens [2]. However, the well maintenance of immune response is metabolically costly and can be hampered without enough energy [3, 4]. Significant alternations could be observed in the metabolic processes of immunocytes during challenge [5, 6]. Various metabolic hormones, such as insulin, were released to modulate energy state in immunocytes, powering the immune events and modulating the immune response [7-9]. In vertebrates, insulin has been reported to modulate the expression of cytokines as well as the growth and proliferation of lymphoid cells directly via binding to its receptors [9, 10]. Other hormones, such as leptin and glucocorticoid, could regulate cellular metabolism as well as modulating immune responses directly [11-13]. However, the immunomodulation function of metabolic hormones in invertebrates is still far from well understood.

Crustacean hyperglycemic hormones (CHHs) are a class of ecdysozoans species-specific pleiotropic hormone with at least one crustacean neurohormone domain [14]. The first CHH was isolated from eyestalks of *Uca pugilator* in 1944 and could significantly up-regulate the hemolymph glucose level [15]. To date, hundreds of CHHs have been identified and they are expressed exclusively in ecdysozoans [16]. It has been found that there could be more than one CHH encoding in an organism, and most of them are expressed in eyestalks [16]. Moreover, those CHHs can be

grouped into two types based on their function or structure [17]. Type I CHH were suggested to elicit the hyperglycemia while type II CHH, with certain modifications on the terminal their peptides, were found regulating either molting or reproduction [16, 18, 19].

Increasing evidences have demonstrated that some members of CHH family are responsive to diverse stimuli. For instance, the expression of CHH increased significantly when shrimps were exposed to heat stress or cold stress [20]. The heavy metal stress could induce a CHH-mediated hyperglycemia in *Palaemon elegans* [21]. Moreover, CHH was found to promote the elimination of *Vibrio harveyi* in shrimp hemolymph and increase the survival rate of infected shrimps, indicating the immunomodulation role of CHH [22]. However, the detailed mechanism of CHH to regulate the immune response is still not clear.

The shrimp *Litopenaeus vannamei* is an important crustacean with great economic value in the world. In the past decades, the shrimp aquaculture suffered from the frequent threatens of various diseases. The white spot syndrome virus (WSSV) is one of the most prevalent and severe pathogens in shrimp aquaculture [23, 24]. It has been reported that the glucose level increased significantly in hemolymph of *L. vannamei* after WSSV infection [24, 25], and two LvCHHs (ADL27417, which was previously suggested as type I CHH and designated as LvCHH I here, and AAN86057 which was suggested as type II CHH and designated as LvCHH II here) with a single or double crustacean neurohormone domain have been identified in shrimp. The purposes of this study were to (1) survey the expression patterns of

LvCHH I and LvCHH II during WSSV infection, (2) verify their modulation role in glucose metabolism, (3) investigate the potential immunomodulation role of LvCHHs by directly binding to the shrimp hemocytes, aiming to provide new hints for the immunomodulation role of glucose-related hormones in invertebrates.

2. Materials and Methods

2.1. Shrimp culture, WSSV preparation and virus challenge.

Shrimps *L. vannamei* (about 12 cm in length) were collected from a commercial farm in Qingdao, China and cultured in aerated seawater at 25°C for 1 week before the experiments.

The WSSV stock was prepared according to the previous report with modifications [26]. Briefly, gills collected from WSSV-infected moribund shrimp were subjected to homogenization and then centrifuged at 3500 g, 4°C for 5 min. The supernatant which contained WSSV particles was diluted with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) before use.

A total of 108 shrimps were randomly divided into two groups for WSSV challenge. The shrimps in the control group received an injection with 25 µL PBS, while shrimps in WSSV challenge group received an injection with 25 µL viral solution (1×10^4 copies per shrimp) diluted in PBS. The shrimps were returned to aerated seawater immediately after treatment. Three shrimps in each group were randomly sampled at 0, 6, 12, 24, 48 and 72 h post injection to collect hemocytes,

eyestalks and gill. The hemolymph samples were extracted from the pericardial cavity with cooled anticoagulant (0.4 M NaCl, 30 mM sodium citrate, 9 mM EDTA) and centrifuged at 800 g, 4°C for 10 min to collect the hemocytes and hemolymph supernatant for glucose determination. The gills were used to quantify WSSV copy numbers, and eyestalks from each group were subjected to RNA extraction and quantitative real-time PCR (qRT-PCR) of LvCHH I and LvCHH II.

2.2. Preparation of recombinant LvCHHs and gain-of-function analysis of LvCHHs *in vivo*

The cDNA fragments encoding the mature peptides of LvCHHs were retrieved from NCBI (<https://www.ncbi.nlm.nih.gov/>) and cloned by gene specific primers P1 and P2 for LvCHH I, P3 and P4 for LvCHH II (Table 1), respectively. The PCR fragments were recombined with pMAL-c5X vector (NEB) for expression in *Escherichia coli* Transetta (Transgen). The rLvCHH I and rLvCHH II with MBP tag were purified via Amylose sepharose column (New England Biolabs) and verified by SDS-PAGE.

The gain-of-function analysis of LvCHHs was conducted *in vivo* by injecting recombinant protein to validate their bioactivity. In brief, a total of 57 shrimps were divided into three groups, and each shrimp in MBP tag, rLvCHH I and rLvCHH II groups received an injection of 0.2 nmol corresponding protein, respectively. At 0, 0.5, 1, 3, 6 and 12 h after injection, three shrimps from each group were sampled for hemolymph supernatant and hemocytes. The hemolymph supernatant was subjected

for glucose determination while hemocytes were employed to detect reactive oxygen species (ROS) level.

2.3. Primary culture of shrimp hemocytes and *in vitro* incubation with rLvCHHs

Primary culture of shrimp hemocytes was conducted with methods described previously with modification [27]. Briefly, hemocytes collected with anticoagulant were immediately centrifuged at 800 *g*, 4°C for 10 min and seeded in 12-well plate with 2 × Leibovitz's L-15 medium (Gibco. Supplemented with 15% FBS, 1% glucose, 5% NaCl, 100 U mL⁻¹ penicillin, and 100 U mL⁻¹ streptomycin). After cultured at 18°C overnight, the hemocytes were checked for viability with microscope and the cells attached to the plate firmly were subjected to further rLvCHHs incubation assay.

About 5 × 10⁵ cells per well seeded in 12-well plate were employed for rLvCHHs incubation. The primary hemocytes seeded in 12-well plate with about 5 × 10⁵ cells per well were incubated with 50 µL of PBS, 0.2 nmol MBP tag, 0.2 nmol rLvCHH I and 0.2 nmol rLvCHH II, respectively at 25°C and sampled at 1 and 3 h later. The cells sampled at 1 h post incubation were first washed three times with modified 2 × Leibovitz's L-15 medium to remove extra rLvCHHs and fixed by 4% paraformaldehyde at room temperature for 30 min for the subsequent immunohistochemistry assay. The cells harvested at 3 h post incubation were employed for RNA extraction and qRT-PCR analysis of shrimp superoxide dismutase (SOD, NCBI accession number: ABC59529), penaeidin-4 (PEN4, NCBI accession number: Q962A7), crustin (NCBI accession number: AY486426) and LvRelish

(NCBI accession number: EF432734) genes.

2.4. Quantification of the WSSV copy number in infected shrimps

Quantification of the WSSV copy number was conducted according to the previous description [28]. The copy number of WSSV in gills was calculated by qRT-PCR with WSSV envelope proteins gene *vp24* (NCBI accession number: AF228518) as indicator and genomic DNA (extraction by Tiangen Marine Animal DNA kit) from gill as template. A standard proliferation curve for copy numbers of WSSV *vp24* was generated using 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , and 10^1 fold copies of *vp24*.

2.5. Determination of the glucose concentration in hemolymph and hemocytes

The glucose concentration was measured by the glucose oxidase enzymatic catalyzation method (Applygen Technologies). Briefly, each sample (5 μ L) was mixed with 195 μ L of glucose reagent in a single well of 96-well plate. After incubation at 37°C for 20 min, the absorbance of the sample was measured at 550 nm with a microplate reader. A standard curve was generated from a set of glucose standards ranging from 0.0 to 1.0 mM. Protein concentration of each sample was determined using BCA Assay Kit (Beyotime) for normalization.

2.6. Determination of intracellular ROS level

The levels of intracellular ROS were detected using the peroxide-sensitive fluorescent probe DCFH-DA (Beyotime). The shrimp hemocytes collected with

anticoagulant agent were incubated with $10 \mu\text{mol L}^{-1}$ DCFH-DA in modified $2 \times$ Leibovitz's L-15 medium at room temperature for 20 min in the dark. The nonfluorescent DCFH-DA freely diffused into cells and was de-esterified by intracellular esterase. After oxidized by intracellular ROS, DCFH-DA is converted to green-fluorescent DCF. The hemocytes pellets were washed for three times and re-suspended in $500 \mu\text{L}$ modified $2 \times$ Leibovitz's L-15 medium before subjected to flow cytometer (BD Bioscience). Relative intracellular oxidative activity is expressed as FITC-A Mean in arbitrary units.

2.7. RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was isolated from the shrimp eyestalks and hemocytes using Trizol reagent (Invitrogen) according to its protocol. The RNase-free DNase I (Promega) was used to digest the genomic DNA from the total RNA, and first-strand cDNA synthesis was carried out based on M-MLV reverse transcriptase using oligo (dT)-adaptor and random primer (Sangon). The reaction was performed at 42°C for 1 h, terminated by heating at 95°C for 5 min. The cDNA mix was diluted to 1:40 and stored at -80°C for subsequent gene cloning and qRT-PCR. The fluorescent quantitative qRT-PCR was carried out in an ABI 7500 Thermal Cycler System. All primers used in the present study were listed in Table 1. The fragment of shrimp 18S gene was employed as internal control. The relative mRNA expression levels were analyzed by the $2^{-\Delta\Delta\text{CT}}$ method [29].

2.8. Preparation of polyclonal antibodies against LvCHHs and western blotting assay

rLvCHH I and rLvCHH II were used for generation of mouse polyclonal antibodies according to the previous report [30]. Briefly, 100 μ L rLvCHHs (1 mg mL⁻¹) were emulsified with 100 μ L complete Freund's adjuvant (Sigma) to immunize 6-8 weeks old mice by subcutaneous implantation. The second and third inoculations were performed on the 14th and 30th day with Freund's incomplete adjuvant (Sigma). The anti-rLvCHHs serum was harvested on the 40th day and stored at -80°C before use.

The efficiency and specificity of polyclonal antibodies against rLvCHHs were verified by western blotting assay. In brief, the rLvCHH proteins were separated by SDS-PAGE and further transferred from gel onto the nitrocellulose membranes (GE Healthcare). The membranes were soaked in blocking buffer with 3% BSA at room temperature for 1 h, followed by the incubation with primary antibodies (diluted at 1:1000) at 4°C overnight. After extensive washing with PBST (135 mmol L⁻¹ NaCl, 4.7 mmol L⁻¹ KCl, 10 mmol L⁻¹ Na₂HPO₄, 2 mmol L⁻¹ NaH₂PO₄, 0.1% Tween-20, pH 7.4), the membranes were incubated with the HRP-linked goat-anti-mouse IgG (Abclonal) for 1 h followed by extensive washing. The immune-blotted protein bands were visualized by SuperSignal West Pico Trial kit (Life Technologies) and developed by X-ray films.

2.9. Tissue section and immunohistochemistry assays of LvCHHs in eyestalks and hemocytes

Tissue section was prepared as previously description with some modifications [31]. The eyestalks of *L. vannamei* were first dissected to remove cuticle and fixed in Bouin's fixative (Saturated picric acid solution: formaldehyde: glacial acetic acid =15:5:1) at room temperature for 24 h. The samples were dehydrated with ethanol and xylene, embedded in paraffin and sectioned (7 mm thick) by RM-2016 microtome (Leika). After rehydrated with ethanol and distilled water, the sections were bathed in 10 mM citrate buffer (2.38 mg L⁻¹ Na₃C₆H₅O₇•2H₂O, 0.4 mg L⁻¹ C₆H₅O₇•H₂O) at 120°C for 6 min and subjected to immunohistochemistry assay. The immunohistochemistry assay for LvCHHs was conducted as following [32]. Eyestalks sections and hemocytes were first blocked in 3% BSA (in PBS) for 30 min, and then incubated with primary rLvCHHs antibodies (1:500 dilution in PBS) at 4°C overnight. The samples were washed by PBST three times before incubated with Alexa Fluor 488-labeled goat-anti-mouse antibody (1:2000 dilution in PBS) at room temperature for 1 h. After washed extensively by PBST, the sections and hemocytes were mounted with 50% glycerin, and monitored with a microscopy (Olympus). Specifically, the sections of eyestalks were stained with 25 mg mL⁻¹ Evans Blue at 37°C for 50 min and hemocytes were incubated with DAPI (Life technology) and DiI (Life technology) to stain the nucleus and cell membrane.

2.10. Statistical analysis

All data were given as means ± SD and subjected to one-way analysis of variance (one-way ANOVA) followed by a multiple comparison (LSD). Differences

were considered significant (labeled with *) at $p < 0.05$ or extremely significant (labeled with **) at $p < 0.01$.

3. Results

3.1. The increase of glucose level and LvCHH transcripts during WSSV infection

WSSV copy number in shrimp gill was determined by qRT-PCR. It remained at low level at 3 and 6 h post injection, increased significantly afterward, and then reached to 4×10^4 copies per ng total DNA of shrimp at 72 h post WSSV challenge. The increase rate of WSSV copy number ascended quickly, reached a peak between 12 and 24 h, and reduced subsequently (Fig. 1, A). The glucose levels in shrimp hemolymph and hemocytes after WSSV challenge were investigated by the glucose oxidase enzymatic colorimetric test. The concentration of glucose in hemolymph did not change significantly in the early 6 h post infection while it increased greatly since 12 h (2.05-fold of that in PBS group, $p < 0.05$) and peaked at 24 h, which was 260 μM per g protein (2.26-fold of that in PBS group, $p < 0.01$) (Fig. 1, B). Afterwards, the glucose levels in hemolymph fell off remarkably at 48 h post challenge (0.52-fold of that in PBS group, $p < 0.05$) (Fig. 1, B). In the hemocytes of shrimps post WSSV injection, the glucose levels declined significantly at 6 h (0.55-fold of PBS group, $p < 0.05$) and increased at 12 and 24 h (1.46-fold and 1.36-fold of that in PBS group respectively, $p < 0.05$). And it dropped again at 48 h, which remained 0.76-fold of that in PBS group ($p < 0.05$) (Fig. 1, C).

The expression levels of LvCHH I and LvCHH II in shrimp eyestalks were

vigorously altered after WSSV challenge. The LvCHH I transcripts increased markedly at 6 h and 12 h (7.56-fold and 2.75-fold of that in PBS group, $p < 0.01$) and recovered to the basal level from 24 to 48 h post injection (Fig. 1, D). Similarly, the expression levels of LvCHH II also increased significantly at 6 and 12 h post challenge (8.53-fold and 2.71-fold of that in PBS group, $p < 0.01$) and dropped to basal state afterward (Fig. 1, E).

3.2. Molecular characters of LvCHHs and the specificity of antibodies

The full-length coding regions of LvCHH I and LvCHH II were cloned using gene-specific primers. Their sequences were consistent with these deposited in NCBI. The cDNAs of LvCHH I and LvCHH II encoded a polypeptide of 114 amino acids or 142 amino acids with canonical signal peptide at the C-terminus, respectively. A classic crustacean neurohormone domain was annotated within LvCHH I from 44 to 112 amino acids (Fig. 2, A), while two crustacean neurohormone domains (31- 66, 72-138 amino acids) were found in LvCHH II (Fig. 2, B). LvCHHs were recombined and expressed in *E. coli* with MBP tags, and rLvCHH I was found to be of 52 kDa (Fig. 2, C), while rLvCHH II was of 59 kDa (Fig. 2, D). The specificities of polyclonal antibodies against LvCHHs were examined by western blotting analysis, and the distinct single bands with the similar molecular weight as rLvCHHs indicated the high specificity and efficiency (Fig. 2, C, D).

3.3 Immunohistochemistry localization of LvCHHs in eyestalks.

Immunohistochemistry assay was performed to detect the localization of endogenous LvCHH I and LvCHH II in shrimp eyestalks. After hematoxylin-eosin staining, neuroendocrine cells were stained dark while neurons were stained light in the eyestalk (Fig. 3, A). LvCHH I and LvCHH II (green) were found sharing the same tissue distribution pattern and dispersed mostly in the dark stained neuroendocrine cells throughout the eyestalk (Fig. 3, B, E), especially in the sinus gland (SG) and the dendritic area of lamina ganglionalis (LG, Fig. 3, C, D, F, G).

3.4. The alternation of glucose and ROS level in hemolymph after injection of rLvCHHs in vivo.

The potential modulation in glucose metabolism by LvCHHs was investigated by its gain-of-function *in vivo*. The glucose levels increased significantly from 0.5 to 3 h in hemolymph of shrimps after rLvCHH I injection (2.36-fold of that in PBS group at 3 h, $p < 0.01$). At the meantime, the glucose levels in LvCHH II group decreased at 0.5 h post injection (0.31-fold of PBS group, $p < 0.01$), and increased significantly at 3 h (1.91-fold of PBS group, $p < 0.01$), but remarkable declined again at 6 h post injection (0.38-fold of PBS group, $p < 0.05$) (Fig. 4, A).

The levels of intercellular ROS were also investigated by detecting the relative fluorescence intensity. The ROS levels in hemocytes of rLvCHH I treated group decreased significantly from 3 to 6 h post injection (0.56-fold of that in PBS group at 3 h, 0.53-fold of that in PBS at 6 h, $p < 0.01$) and then recovered to the basal level at 12 h. However, no significant difference was observed between rLvCHH II treated

group and control group (Fig. 4, B).

3.5. The interaction between LvCHHs and shrimp hemocytes.

The ability of LvCHHs to bind to shrimp hemocytes was verified by immunohistochemistry assay. The positive signal was indicated by green fluorescence while the membrane and nucleus of shrimp hemocytes was in red and blue fluorescence, respectively. After rLvCHH I and rLvCHH II incubation, significant positive signal of FITC labeled antibodies was detected on the membrane of shrimp hemocytes, while no positive signal was observed in the MBP tag group (Fig. 5).

3.6. The induction of immune-related genes expression by LvCHHs in shrimp hemocytes.

To further investigate the immunomodulation roles of LvCHHs, the primary culture shrimp hemocytes were incubated with rLvCHHs and expression alternations of immune-related genes, including SOD, LvRelish and two antimicrobial peptides (AMPs, PEN4 and crustin) were detected by qRT-PCR. It turned out that the expression levels of these genes could be modulated discriminately by either LvCHH I or LvCHH II. In detail, the expression level of SOD mRNA was up-regulated dramatically at 3 h post rLvCHH I incubation, which was 4950.91-fold of that in MBP tag group ($p < 0.01$) (Fig. 6, A). The expression levels of LvRelish as well as that of PEN4 and crustin were also significantly induced after the incubation, which altered to 409.39, 552.61 and 3060.73-fold of control group respectively ($p < 0.01$) (Fig. 6, B,

C, D). On the contrary, no significantly changes of the SOD and LvRelish mRNA were observed in rLvCHH II incubation group compared with MBP tag group (Fig. 6, A, B). The expression of PEN4 and crustin mRNA, at the mean time, increased to 4.15-fold and 4.19-fold of that in MBP tag group, respectively ($p < 0.01$) (Fig. 6, C, D).

4. Discussion

A robust immune system is crucial for host to survive from pathogen infection, which will cost considerable amount of energy [33]. The glycogen degradation and glucose uptake are usually accelerated after infection to generate more energy in immunocytes [1, 34]. It has been reported that the glucose concentration in hemolymph of crustacean increased significantly after lipopolysaccharide injection [35, 36]. In the present study, a significant increase of hemolymph glucose level was observed during WSSV challenge (Fig. 1, B), indicating the glycogen degradation was accelerated after WSSV infection. Meanwhile, the glucose in hemocytes was increased firstly and then declined after infection (Fig. 1, C), suggesting the increase of both glucose consumption and uptake during the immune response against WSSV infection. These results were consistent with the previous reports that the glucose metabolism in shrimps was dynamically modulated after WSSV infection [25, 37].

There are two major hormones (insulin and glucagon) responsible for glucose metabolism in vertebrates [38], but either of them has been identified in shrimp *L. vannamei*. So far, multiple CHHs were identified in the shrimp and suggested to

modulate the glucose metabolism. In the present study, the expression alternations of LvCHH I and LvCHH II, two members of CHH family previously identified in *L. vannamei* and encoded with one or two crustacean neurohormone domains (Fig. 2, A, B), were surveyed during WSSV infection. The transcripts of both LvCHH I and LvCHH II in shrimp eyestalks were drastically up-regulated after WSSV challenge (Fig. 1, D, E), which was consistent with the alternations of hemolymph glucose (Fig. 1, B). Moreover, the positive signals of these two LvCHH were observed in the LG and the SG of eyestalks (Fig. 3). These results indicated that different peptides of CHH family might be co-expressed in the same site of endocrine system and function crucially in shrimps during WSSV infection.

It has been suggested that CHHs could be classified into two types (type I and type II) and the type I CHHs could induce glucose level significantly in most crustaceans, such as crab *Carcinus maenas* and crayfish *Orconectes limosus* [39]. The gain-of-function assay was subsequently conducted to investigate the modulation of hemolymph glucose concentration by rLvCHH I or rLvCHH II stimulation. As a result, the glucose levels in LvCHH I injection group were significantly promoted since 0.5 h post injection and peaked at 3 h, which dropped to basal level afterward (Fig. 4, A). Meanwhile, the glucose level fluctuated vigorously in LvCHH II group, which decreased significantly at 0.5 h and 6 h post injection and increased at 3 h. It was therefore suggested that LvCHH I could act canonically as type I CHHs promoting the glucose level *in vivo* quickly and vigorously, while the modulation by LvCHH II was more complicated. Intracellular ROS has been suggested as the

by-product of glucose metabolism in most organisms and its level could be up-regulated when glucose concentration increased continuously [40, 41]. Beyond expectation, the ROS level in rLvCHH I injection group decreased significantly (Fig. 4, B). As observed in mammals, ROS level could be repressed directly by some hormones such as glucocorticoids [42]. Therefore it was speculated that LvCHH I could trigger downstream signals involved in ROS clearance in shrimp hemocytes besides glucose modulation. At the meantime, the ROS level remained unchanged in LvCHH II group (Fig. 4, B), indicated the absence of LvCHH II-mediated modulation in ROS clearance.

Considering that most hormones activate downstream pathways via binding to the target cells directly [43], the direct modulation role of LvCHHs on shrimp hemocytes were subsequently investigated by *in vitro* incubation. The immunochemistry assay of LvCHHs was conducted after incubation with primary shrimp hemocytes to investigate the direct binding on cell membrane. As a result, positive signals of both LvCHHs were observed in the cell membrane after their incubation (Fig. 5), which for the first time demonstrated that LvCHHs could directly bind to cytomembrane of shrimp hemocytes. Meanwhile, the expression level of shrimp SOD gene, a crucial scavenger of intracellular ROS, was significantly up-regulated after rLvCHH I incubation instead of rLvCHH II incubation (Fig. 6, A). This result was consistent with the alternations of ROS levels in gain-of-function assay of LvCHH I *in vivo*, confirming its modulation role in ROS clearance and suggesting the protective role against oxidative stress caused by increased glucose

metabolism in shrimp during pathogen infection [25, 44]. Besides of the modulation on ROS level, it was interesting to know other downstream effects by LvCHHs, especially the induction of immune-related genes. In vertebrates, glucose-regulating hormone insulin was found targeting NF- κ B pathway, which was crucial in activating the transcription of multiple immune genes [8, 9]. Meanwhile, the insulin signal pathway could modulate the expression of two AMPs (*Diptericin* and *Drosomycin*) directly in *Drosophila melanogaster* [45]. In the present study, a significant increase of LvRelish transcripts, subunit of NF- κ B gene, was observed in shrimp hemocytes incubated with rLvCHH I but not rLvCHH II (Fig. 6, B), indicating that LvCHH I could trigger the activation of NF- κ B signal pathway. In addition, the mRNA levels of two shrimp AMPs (PEN4 and crustin) were found significantly up-regulated after either rLvCHH I or rLvCHH II stimulation while that in LvCHH I group was remarkably higher (Fig. 6, C, D). Collectively, those results demonstrated that LvCHHs might contribute greatly to the robust immune response of host by powering the hemocytes as well as activating the expression of immune-related genes directly.

Above results have demonstrated the differentiated effects between LvCHH I and LvCHH II in glucose metabolism and immune response. As known, the function of certain protein is decided by its structure. The structural differences of LvCHH I and LvCHH II (Fig. 2, A, B) might explain above modulation discriminations. Meanwhile, little information was known about the potential receptors of LvCHHs [17], which might also dedicate to the modulation differences and should be investigated in future study. In conclusion, above results demonstrated the direct immunomodulation role of

LvCHHs in crustaceans for the first time and would shed new light into understanding the interaction between glucose-regulating hormone and immune response in crustaceans.

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Figure legend

Fig. 1. The increase of glucose levels and LvCHH transcripts during WSSV infection

(A) The copy number of WSSV *vp24* gene was investigated to indicate replication of WSSV during infection. (B) The hemolymph glucose concentration increased significantly during the infection. (C) The concentration of glucose in hemocytes was vigorously modulated during WSSV challenge. (D) Expression levels of LvCHH I and LvCHH II increased remarkably at 6 h and 12 h post injection. Statistical

significance is indicated by single ($p < 0.05$) or double ($p < 0.01$) asterisks.

Fig. 2. The domain characters of LvCHHs and specificity for their polyclonal antibodies.

(A) Protein domains of LvCHH I and LvCHH II were predicted by SMART (<http://smart.embl.de/>). Signal peptides (the red area) were observed in either hormone while LvCHH I encoded a canonical “Crust_neurohorm” domain and LvCHH II encoded both “Crust_neuro_H” domain and “Crust_neurohorm” domain. (B) SDS-PAGE and Western-blot analysis of rLvCHH I and rLvCHH II. Lane 1: standard protein molecular weight marker; lane 2: induced rLvCHHs; lane 3: negative control (without induction); lane 4: purified rLvCHHs; lane 5: Western-bolt of rLvCHHs with anti-rLvCHHs-antibodies.

Fig. 3. Localization of LvCHHs in eyestalk by immunohistochemistry.

(A) Hematoxylin and eosin stained shrimp eyestalk (LG, the lamina ganglionalis; SG, the sinus gland; ME, the medulla externa; MI, the medulla interna; MT, medulla terminalis. Bar 200 μ m). (B) Significant signals of LvCHH I protein (green) were observed in the SG and LG region of eyestalk (Bar 100 μ m). (C) Magnified micrograph of LvCHH I in the SG region (Bar 50 μ m). (D) Magnified micrograph of LvCHH I in the LG region (Bar 50 μ m). (E) LvCHH II protein (green) was also observed in the SG and LG region of eyestalk (Bar 100 μ m). (G) Magnified micrograph of LvCHH II in the SG region (Bar 50 μ m). (F) Magnified micrograph of

LvCHH II in the LG region (Bar 50 μ m).

Fig. 4. LvCHHs modulated glucose and ROS level *in vivo*.

(A) Hemolymph glucose level in shrimps injected with rLvCHH I or rLvCHH II. (B) Intracellular ROS level in shrimp hemocytes after rLvCHH I or rLvCHH II injection. Statistical significance is indicated by single ($p < 0.05$) or double ($p < 0.01$) asterisks.

Fig. 5. The interaction between LvCHHs and shrimp hemocytes.

rLvCHHs (green) were found binding to the cell membrane of shrimp hemocytes (red for cell membrane, blue for the nucleus).

Fig. 6. LvCHHs modulated the expression of immune-related genes.

(A) SOD transcripts were significantly induced in shrimp hemocytes after rLvCHH I incubation instead of rLvCHH II incubation. (B) Significant increase of LvRelish transcripts was observed in shrimp hemocytes after rLvCHH I stimulation. (C) The transcripts of PEN4 increased significantly after either rLvCHH I or rLvCHH II stimulation. (D) The transcripts of crustin increased remarkably in hemocytes after rLvCHHs incubation. Statistical significance is indicated by single ($p < 0.05$) or double ($p < 0.01$) asterisks.

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A **B** **C** **D**

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Table. 1. Nucleotide sequence of primers used in this study.

Primer	Sequence(5'-3')
Oligo(dT)-adaptor	GGCCACGCGTCGACTAGTACT ₁₇
Clone primers	
P1 (forward)	ACCAAACGCTCGCTCTTC
P2 (reverse)	TTTCCCGACCATCTGGAC
P3 (forward)	CGCTCCGTCGACGGCG
P4 (reverse)	CTTCCCTACCAGCTGGACGG
RT primers	
18s (forward)	TATACGCTAGTGGAGCTGGAA
18s (reverse)	GGGGAGGTAGTGACGAAAAAT
vp24 (forward)	CCAGTTCAGAATCGGACGTT
vp24 (reverse)	AAAGACGCCTACCCTGTTGA
LvCHH I (forward)	CTGAATGCAGAAGTAACTGTTACAAC
LvCHH I (reverse)	CTATTTCCCGACCATCTGGAC
LvCHH II (forward)	ACACCTTCGACCACTCCTGC
LvCHH II (reverse)	GTAGCAGTTACTCTTGCACTCGGT
PEN4 (forward)	CGATACCCAGGCCACCA
PEN4 (reverse)	GGAATATCCCTTTCCACGT
Crustin (forward)	GGAGGGTCAAGCCTACTGC
Crustin (reverse)	ACGTGGGCATGTGGGAC
LvRelish (forward)	CCATACTCAGACCACTCAGC
LvRelish (reverse)	TGAATGCTCCATCGTGGT
LvSOD (forward)	GACTGGTTCCGTTGCTTGGC
LvSOD (reverse)	TCTCATAACGCTCATTACG