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The immunomodulatory function of invertebrate specific neuropeptide FMRFamide in oyster *Crassostrea gigas*

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ABSTRACT

As one of the most important neuropeptides identified only in invertebrates of Mollusca, Annelida and Arthropoda, FMRFamide (Phe-Met-Arg-Phe-NH₂) involves in multiple physiological processes, such as mediating cardiac frequency and contraction of somatic and visceral muscles. However, its modulatory role in the immune defense has not been well understood. In the present study, an FMRFamide precursor (designed as CgFMRFamide) was identified in oyster *Crassostrea gigas*, which could be processed into nineteen FMRFamide peptides. Phylogenetic analysis revealed that CgFMRFamide shared high similarity with other identified FMRFamides in mollusks. The mRNA of CgFMRFamide was mainly concentrated in the tissues of visceral ganglia, hepatopancreas and hemocytes, and a consistent distribution of FMRFamide peptide was confirmed by immunohistochemistry and immunocytochemistry assays. The mRNA expression level of CgFMRFamide in hemocytes was significantly up-regulated after immune stimulation with lipopolysaccharide (LPS). After the concentration of FMRFamide was increased by exogenous injection, the *in vivo* expressions of pro-inflammatory cytokine CgIL17-5, as well as the apoptosis-related CgCaspase-1 and CgCaspase-3 in hemocytes were promptly increased ($p < 0.05$), but the concentration of signal molecule nitric oxide (NO) was significantly down-regulated ($p < 0.05$). Meanwhile, an increased phosphorylation of p38 MAP kinase in hemocytes was also detected after the FMRFamide injection. These results collectively demonstrated that the conserved FMRFamide could not only respond to immune stimulation, but also regulate the expression of immune effectors and apoptosis-related genes, which might be mediated by p38 MAP kinase pathway, thereby effectively involved in clearing pathogens and maintaining homeostasis in oysters.

1. Introduction

Signal molecules, such as neurotransmitters, neuropeptides and endocrine hormones, can be released from the nervous or/and endocrine systems to regulate various physiological activities and keep the homeostasis of organisms [1]. Neuropeptides are small protein-like molecules (peptides) and the most ancient signals, which are ubiquitous in the animal kingdom from cnidarians to human [2,3]. As one of the neuropeptides, Phe-Met-Arg-Phe-NH₂ (FMRFamide) was the first identified from the clam *Macrocallista nimbosa* [4], and it was subsequently found to exist only in invertebrates specifically. At present, the authentic FMRFamide has been found in three phyla [5], Annelida [6],

Arthropoda [7], and most in Mollusca [8–10]. The common model of those identified FMRFamide precursors contains a unique tetrabasic site corresponding to a furin-processing site (RXK/RR) that typically separates the precursor into two domains: N-terminal region encoding a signal peptide, the FL/IRFamide peptides and a decapeptide, while the C-terminal domain harbors the FMRFamides [11]. Those FMRFamide precursors can be processed into mature peptides by proteases, and then released to function in various physical processes.

Recently, several FMRFamides have been identified in various species, and their physical functions have also received much attention. For example, in the nervous system of *Aplysia*, FMRFamide was reported to modulate the synaptic plasticity through activation of p38

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mitogen-activated protein (MAP) kinase [12]. In *Cepaea nemoralis*, FMRFamide could enhance the neuronal nitric oxide synthesis in its nervous system [13]. Moreover, FMRFamide was not only concentrated in the nervous system, but also equally localized and functioned in the peripheral organs. For example, in the siphon and adjacent mantle region of *Dreissena polymorpha*, FMRFamide was reported to regulate the contraction of muscles [14]. In the digestive gland of *Helix lucorum*, FMRFamide could protect cell against apoptosis through inhibiting the cells mitosis [15]. FMRFamide was also found in hemolymph of *Macrallista* at a concentration of 10^{-9} M, which could excite the isolated *Macrallista* ventricles [16]. However, the reports about the possible immunomodulatory function of FMRFamide in the hemolymph are still very limited.

The Pacific oyster *Crassostrea gigas* is one of most important marine mollusks and contributes greatly to the aquaculture industry worldwide. Living in the estuarine intertidal zone, oysters have well-developed stress tolerance mechanisms to adapt to the fluctuating environment and high-loaded pathogens [17]. Neuro-endocrine-immunity (NEI) system, regarded as the vital “super-systems”, is engaged in multiple physical adaptations in response to stress [18]. Investigations of the components and functions of NEI system in oyster *C. gigas* will shed light on the adaptation mechanism of marine invertebrates. In the present study, an invertebrate-specific neuropeptide FMRFamide (CgFMRFamide) was identified from *C. gigas* with the purposes to (1) analyze its molecular characteristics, (2) explore the distribution of its mRNA and peptide in different tissues, (3) investigate its response after immune stimulation, and (4) survey its immunomodulatory role in the immune system of oysters.

2. Materials and methods

2.1. Oysters

Pacific oysters *C. gigas*, about 2 years, were collected from a local farm in Dalian, Liaoning province, China. Oysters were maintained in tanks filled with filtered and aerated seawater (about 15–20 °C), and fed with condensed microalgae once a day for at least one week before processing.

2.2. LPS stimulation

One hundred oysters were randomly selected and sawed in the closed side of the shell adjacent to the adductor muscle. After acclimated for another one week, those oysters were randomly divided into two groups on average, which received an injection of 100 µL phosphate buffer saline (PBS) (1.5 mM KH_2PO_4 , 8 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.14 M NaCl, 3 mM KCl, pH 7.4) and 100 µL lipopolysaccharide (LPS, 0.5 mg mL⁻¹) (Sigma, USA) dissolved in PBS, respectively. The oysters were returned to filtered seawater immediately after treatment. Nine individuals were randomly sampled at 0, 6, 12, 24 and 48 h post injection. The hemolymph collected from three oysters were pooled together as one sample, and three parallel samples for each time points. The hemolymph was collected from sinus of different treated oysters with 10 mL syringe, and centrifuged at 800 g, 4 °C for 10 min to harvest hemocytes. Those enriched hemocytes were dissolved in Trizol reagent, immediately frozen with liquid nitrogen, stored at –80 °C, and prepared for RNA isolation. The tissues including adductor muscle, gills, gonad, hemocytes, mantle, visceral ganglia and hepatopancreas collected from untreated oysters were also kept in Trizol reagent at –80 °C for RNA isolation.

2.3. FMRFamide treatment

Eighty oysters were randomly selected and equally divided into seawater (SW) group and FMRFamide (FM) group. Oysters in SW group received an injection with 100 µL filter sterilized (0.22 µm pore size)

Table 1
Primers used in this study.

Primer name	Sequence (5'-3')
Clone primers	
P1 (oligo dT-adaptor)	GGCCACGCGTCGACTAGTACT
P2 (CgFMRFamide-forward)	ATGGGGACGTGGACATAC
P3 (CgFMRFamide-reverse)	TCATACAGATTTTCCGAATCGC
Sequence primers	
P4 (M13-47)	CGCCAGGGTTTTCCCACTCAGCAG
P5 (RV-M)	GAGCGGATAACAATTTCACACAGG
RT primers	
P6 (CgIL17-5-RT-forward)	TGTCGTTGCTCTACCATGAT
P7 (CgIL17-5-RT-reverse)	CGTCCTTGCCCTTACTGACTAGA
P8 (CgCaspase-1-RT-forward)	ACAACCTGAAAGCAGAGGAAAT
P9 (CgCaspase-1-RT-reverse)	CGGCAAGCCTGGATAAAGAA
P10 (CgCaspase-3-RT-forward)	GGCTGACTTTCTGATTGCTT
P11 (CgCaspase-3-RT-reverse)	ATGTCGGAGTGGGAGGTGTT
P12 (CgEF-RT-forward)	AGTCACCAAGGCTGCACAGAAAG
P13 (CgEF-RT-reverse)	TCCGACGTATTCTTTGCGATGT
P14 (CgFMRFamide-RT-forward)	TTCTTTCTAACTGGTTTCACT
P15 (CgFMRFamide-RT-reverse)	TTATGACTTTGTTACAGAGCCT

seawater, and the oysters in FM group received an injection with 100 µL FMRFamide (100 µM in seawater). The concentration of FMRFamide adopted to oyster injection experiment was determined according to the reports in *Helix lucorum* and *Helix aspersa* [15,19]. The hemolymph was collected from nine oysters (three oysters as one parallel) in each treatment at 0, 1, 6 and 12 h after injection, respectively. The hemolymph supernatant and hemocytes were harvested after centrifugation, and the hemolymph supernatant was immediately stored at –80 °C for NO concentration measurement and the hemocytes were kept in Trizol reagent at –80 °C for RNA extraction.

2.4. Total RNA extraction and real-time PCR analysis

Total RNA from different tissues of oyster was extracted using Trizol (Invitrogen, USA) following manufacturer's instruction. The cDNA synthesis was performed by one-step gDNA removal and cDNA synthesis supermix kit (Transgen, China) based on the manufacturer's instruction using the total RNA as template and oligo (dT)-adaptor as primer P1 (Table 1). The reaction was performed at 42 °C for 30 min and terminated by heating at 85 °C for 5 s. The cDNA mix was stored at –80 °C for quantitative real-time PCR and gene cloning.

The mRNA expression of target genes was determined by real-time PCR (RT-PCR) with ABI Quantstudio sequence detection system according to the manual (Applied Biosystems, USA). Specific primers for target genes were listed in Table 1 and two specific primers, P12 and P13, were used to amplify part of elongation factor (EF) (LOC105338957) in oysters as an internal control. After real-time PCR cycle, dissociation curve was performed to confirm that only one PCR product was amplified and detected. The $2^{-\Delta\Delta\text{CT}}$ method was used to analyze the expression level of genes, and all data were given in terms of relative mRNA expression of mean $\pm$ S.E. (N = 3).

2.5. Gene clone and sequence analysis

A pair of specific primers P2 and P3 (Table 1) were designed according to the nucleotide sequence of CgFMRFamide (GeneBank Accession NO. [XM_011445484.2](https://www.ncbi.nlm.nih.gov/sequence/annotation/?term=XM_011445484.2)) acquired from National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>), and the PCR product amplified by this pair primers was gel-purified, cloned into the pMD19-T simple vector (Takara, Japan), and sequenced with primers P4 and P5 (Table 1). The deduced amino acid sequence of CgFMRFamide was analyzed with the Expert Protein Analysis System (<http://www.expasy.org>) and the protein domain was predicted with the simple modular architecture research tool (SMART) version 8.0 (<http://www.smart.emblheidelberg.de/>). The homology

amino acid sequences of CgFMRFamide from other species were downloaded from NCBI database, and used to create the multiple sequence alignment by ClustalW Multiple Alignment program (<http://www.ebi.ac.uk/clustalw/>). A phylogenetic tree was estimated in Mega 6.06 by maximum-likelihood (ML) based on the JTT matrix-based model.

2.6. Immunohistochemical and immunocytochemical assay

Immunohistochemical assay was performed according to the previous description with little modification [20]. Fresh tissues of hepatopancreas, adductor muscle, gills, visceral ganglia, mantle and gonad were collected from untreated oysters. They were fixed in Bouin's fluid (Sangon, China) at room temperature for 24 h, faded in 70% ethanol until nearly colorless, and sequentially dehydrated in 80%, 95%, 100% ethanol and xylene. The samples were then embedded in paraffin wax, sectioned (7 μ m) with rotary microtome (Leica, Germany), and mounted on slides. After dewaxing and rehydration, the antigens retrieval was performed in sodium citrate-hydrochloric acid buffer and the tissues on the slides were permeabilized with 0.5% Triton-100 for 5 min. After washing with PBST (PBS containing 0.05% Tween-20), the slides were blocked with 3% BSA at 37 °C for one hour, incubated with commercial antibody of FMRFamide (Abcam, UK, diluted 1:1000 in 3% BSA) at 37 °C for another one hour (incubated without antibody of FMRFamide set as negative control), washed in PBST, and then incubated with Alexa Fluor 488-labeled goat-anti-rabbit secondary antibody (Abcam, UK, diluted 1:2000 in 3% BSA) at 37 °C for 50 min. After washing with PBST, the slides were incubated with 0.5% Evan's blues (Solarbio, China, diluted in PBST) for 10 min and thoroughly washed with PBST. Finally, cover slides were mounted after 50% glycerin added on the slides, and the fluorescence was observed under fluorescence microscope (Axio Imager A2, Zeiss).

Hemolymph was collected from six oysters and directly seeded on slides covered with polylysine. Four hours later, the supernatant was discarded and the hemocytes were fixed with 4% paraformaldehyde (PFA) for 15 min. After washing with TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20), the hemocytes were permeabilized with 0.5% Triton-100 for five minutes, blocked with 3% BSA in TBST at room temperature for one hour, and incubated with the commercial antibody of FMRFamide (diluted 1:1000 in 3% BSA) at room temperature for one hour (incubated without antibody of FMRFamide set as negative control). After washing with TBST, the hemocytes were incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Abcam, UK, diluted 1:2000 in 3% BSA) for one hour. Finally, the hemocytes were washed three times with TBST before incubation with DAPI (Beyotime, China, diluted 1:10000 in PBS) for five minutes, and observed under fluorescence microscope (Axio Imager A2, Zeiss).

2.7. NO concentration measurement

The concentrations of NO in hemolymph supernatant were measured according to the instruction of total nitric oxide assay kit (Beyotime, China) with Griess reaction method [21]. After reducing by nitrate reductase, the concentrations of nitrite and nitrate were measured, and the concentration of NO in oyster hemolymph was calculated according to the standard curves of NaNO₂.

2.8. Western blot assay

The hemocytes were collected from ten oysters in each group after stimulated with seawater and FMRFamide for one hour as described as above. The hemocytes were lysed with SDS lysis buffer (Beyotime, China) containing inhibitors of protease and phosphatase at 4 °C for 15 min. The cell lysate was collected after centrifugation at 12000 g, 4 °C for 10 min. The protein concentration was determined by BCA protein assay kit (Beyotime, China). Thirty micrograms of protein from

different stimulation groups were separated by SDS-PAGE, and the proteins in the gel were transferred onto nitrocellulose membrane. After blocking by 3% BSA in TBST at 37 °C for two hours, the membrane was incubated with the antiserum against phospho-p38 MAP kinase (Cell signaling technology, USA, diluted 1:1000 in 3% BSA) or β -tubulin (Transgen, China, diluted 1:1000 in 3% BSA) at 37 °C for another two hours. The commercial monoclonal antibody of phospho-p38 MAP kinase was produced by rabbits with a synthetic phosphopeptide corresponding to residues surrounding Thr180/Tyr182 of human p38 MAPK, and the amino acid sequence of human p38 MAPK site (MTGYVAT-RWY) was identical to the phospho-P38 sites of oyster [22]. Following thoroughly washed with TBST for three times, the membrane was incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Abcam, UK, diluted 1:2000 in 3% BSA) at 37 °C for one hour. After thoroughly washing with TBST for another three times, the immune-reactive protein bands were visualized by using BCIP/NBT alkaline phosphatase color development kit (Beyotime, China) in the dark for 30 min, and imaged by Amersham Imager 600 (GE Healthcare).

2.9. Statistical analysis

All data were presented as mean $\pm$ S.D., and analyzed by statistical package for social sciences (SPSS) 16.0. Statistical differences between samples were estimated using ANOVA (Duncan's test) and *t*-test, and differences were considered statistically significant at $p < 0.05$ and extremely significant at $p < 0.01$.

3. Results

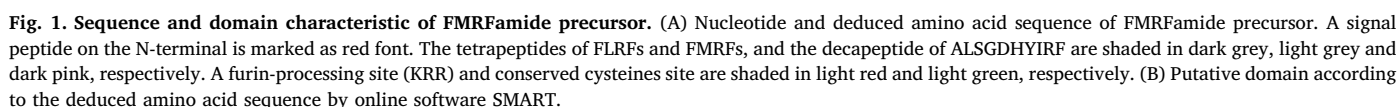
3.1. The sequence characteristics and phylogeny relationship of CgFMRFamide

According to the genome annotation, only one FMRFamide precursor gene (CgFMRFamide) was screened from oyster *C. gigas* genome. After amplifying with specific primers, the 1702 bp open reading frame of CgFMRFamide was obtained from cDNA library, which encoded a polypeptide of 432 amino acids with a predicted molecular mass of 51.7 kDa and a theoretical isoelectric point of 9.65. The deduced amino acid sequence of CgFMRFamide contained two FLRFamide, a decapeptide ALSGDHYIRFamide, and nineteen FMRFamides (Fig. 1A). SMART analysis revealed that CgFMRFamide contained a signal peptide from 1 to 24 amino acid and twenty-two tandemly repeat FARP domains (Fig. 1B).

A phylogenetic tree was constructed to analyze the evolutionary relationship of FMRFamide precursor peptides from different species using maximum-likelihood (ML) method. FMRFamides in the phylogenetic tree were all defined in invertebrates and divided into three phyla of Mollusca, Annelida and Arthropoda. The CgFMRFamide was firstly clustered with FMRFamides from *Mytilus edulis* and *Mizuhopecten yessoensis*, and then gathered with those from gastropoda of *California sea hare*, *Aplysia californica*, *Deroceras reticulatum*, *Lymnaea stagnalis*, *Haliotis asinina* and cephalopoda of *Sepia officinalis* to form Mollusca branch. The FMRFamides in Mollusca branch gathered with that from *Platynereis dumerilii* in Annelida, and finally clustered with those from *Drosophila melanogaster* and *Musca domestica* in Arthropoda (Fig. 2).

3.2. The mRNA expression level of CgFMRFamide precursor gene in different tissues

The relative mRNA expression level of CgFMRFamide was determined in different tissues using RT-PCR with gonad as reference. The CgFMRFamide transcripts were ubiquitously detectable in all tested tissues, including hepatopancreas, hemocytes, gonad, visceral ganglia, gills, mantle, and adductor muscle. The highest expression of CgFMRFamide mRNA was found in visceral ganglia, which was about 286.6-fold ($p < 0.01$) of that in gonad (Fig. 3). The expression levels in



Immunohistochemistry and immunocytochemistry assays were employed to detect the distribution of FMRFamide peptide in different tissues of oyster, including hepatopancreas, adductor muscle, gills, visceral ganglia, mantle, gonad, and hemocytes. The green positive signals were observed in the digestive tubules of hepatopancreas, visceral ganglia and margin of mantle (Fig. 4A) under fluorescence microscope, while no positive signal was detected in other examined tissues and negative control. FMRFamide peptide was mainly located in the cytoplasm of oyster hemocytes (Fig. 4B).

In order to explore the immune regulatory activities of FMRFamide, the commercial FMRFamide was injected into oysters' adductor muscles, and the temporal mRNA expression level of CgIL17-5 in hemocytes and the concentration of NO in hemolymph supernatant were measured

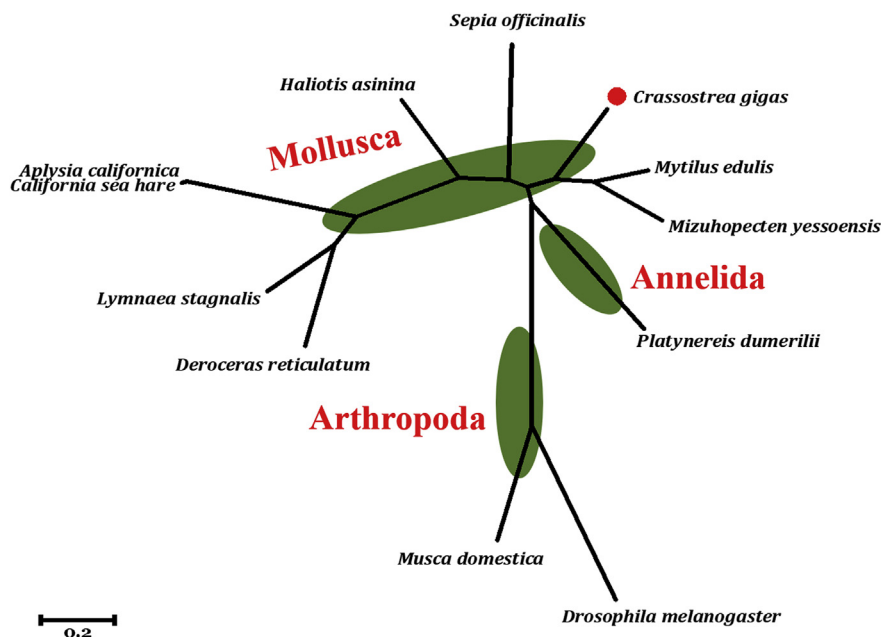


Fig. 2. Maximum likelihood (ML) phylogenetic tree based on the amino acid sequences of different FMRFamides precursor. The scale bar represented the conversion of branch length to genetic distance between clades (0.2 = 20% genetic distance). The FMRFamides used for phylogenetic analysis were from *Crassostrea gigas* (XP_011443786.1), *Mytilus edulis* (CAA10949.1), *Mizuhopecten yessoensis* (XP_021378855.1), *California sea hare* (A25790), *Aplysia californica* (AAA27752.1), *Deroceras reticulatum* (ARS01392.1), *Lymnaea stagnalis* (AAA63280.1), *Haliothis asinine* (ACD65487.1), *Sepia officinalis* (P91889.1), *Platynereis dumerilii* (AEE25641.1), *Drosophila melanogaster* (AAA28536.1) and *Musca domestica* (A6P3B2.1).

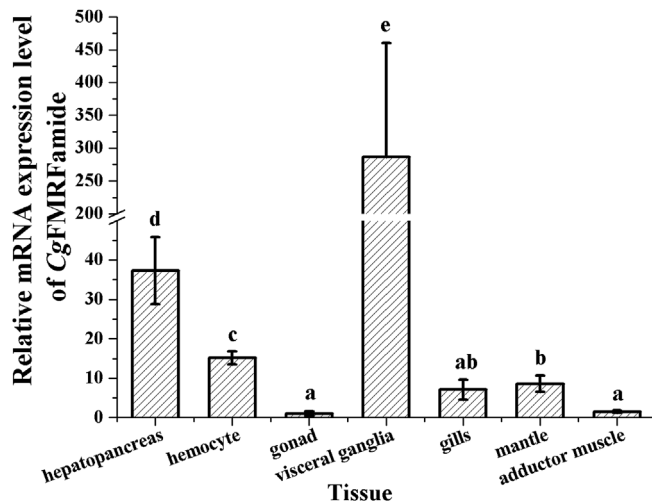


Fig. 3. The mRNA expression of CgFMRFamide in different tissues of *C. gigas*. Data were represented as the ratio of CgFMRFamide mRNA level to that of gonad and normalized to that of CgEF. Vertical bars represented the mean $\pm$ S.D. (N = 3) and the significant letters (a, b, etc) indicated significant differences ($p < 0.05$, ANOVA).

after FMRFamide stimulation. The expression of CgIL17-5 responded rapidly and began to increase at 1 h post injection. The expression of CgIL17-5 significantly up-regulated at 1 h and 6 h, which was 57.1-fold and 6.3-fold higher than that of in seawater group, respectively ($p < 0.01$), and recovered to normal level at 12 h (Fig. 6A). Meanwhile, a continuous down-regulation of the NO concentration in hemolymph was detected during 1–12 h after the oysters received an injection with FMRFamide ($p < 0.01$) (Fig. 6B).

The mRNA expression levels of apoptosis-related CgCaspase-1 and CgCaspase-3 were also determined after FMRFamide stimulation. The expressions of CgCaspase-1 and CgCaspase-3 both increased rapidly at 1 h, which was 2.0-fold and 6.7-fold, respectively ($p < 0.01$), and then recovered to the initial level at 6 h after FMRFamide stimulation (Fig. 7). Those results indicated FMRFamide could induce the expression of CgCaspase-1 and CgCaspase-3.

3.6. The phosphorylation of p38 MAP kinase after injection with FMRFamide

The phosphorylation of p38 MAP kinase was detected by western blotting at one hour after the oysters received an injection with FMRFamide. The relative phosphorylated level of p38 MAP kinase with molecular mass about 40 kDa was significantly increased after stimulation with FMRFamide (1.89-fold of that in the seawater group, $p < 0.05$) compared with that in seawater treatment group, while no significant change of the β -tubulin with molecular mass about 55 kDa was observed after stimulation, which indicated the FMRFamide might activate p38 MAP kinase pathway (Fig. 8).

4. Discussion

Neuroendocrine-immune (NEI) network is essential for homeostasis during stress and infection, which regulates a balanced immune response to effectively clear the pathogen and minimize the damage to the host [23]. Neuropeptides is one kind of most important neuromodulators in NEI system, which has been proved to exert immunomodulatory function in both vertebrates and invertebrates. For example, neuropeptide Y was responsible for the production of the pro-inflammatory cytokines IL-12 and TNF- α in mice [24]. Enkephalins exhibited strong antibacterial activity and stimulated the phagocytosis of immunocyte as well as the secretion of cytokines in *Channa punctatus* [25,26]. The immunomodulatory role of neuropeptides was also reported in invertebrates. Neuropeptide Y could inhibit the chemotaxis of immunocytes in *Mytilus edulis* [27] and [Met⁵]-enkephalin could modulate the phagocytic and antibacterial functions of hemocytes in *Crassostrea gigas* [28]. However, there is still no report about the immune function of FMRFamide, which is specifically existed in invertebrates.

In the present study, an FMRFamide precursor gene was identified and cloned from oyster *C. gigas*. In the genome information of *C. gigas*, only one FMRFamide precursor gene was annotated, which was different from that in the gastropods of *Haliothis asinine* with two FMRFamide precursor genes [10]. The deduced amino acid sequence of FMRFamide precursor contained a signal peptide at N-terminal, two conserved FLRFamide, a typical decapeptide ALSGDHYIRFamide and nineteen classical FMRFamides. Because no FMRFamide had not been identified in vertebrates [5], all FMRFamide precursor peptides in the phylogenetic tree were defined in invertebrates and divided into three

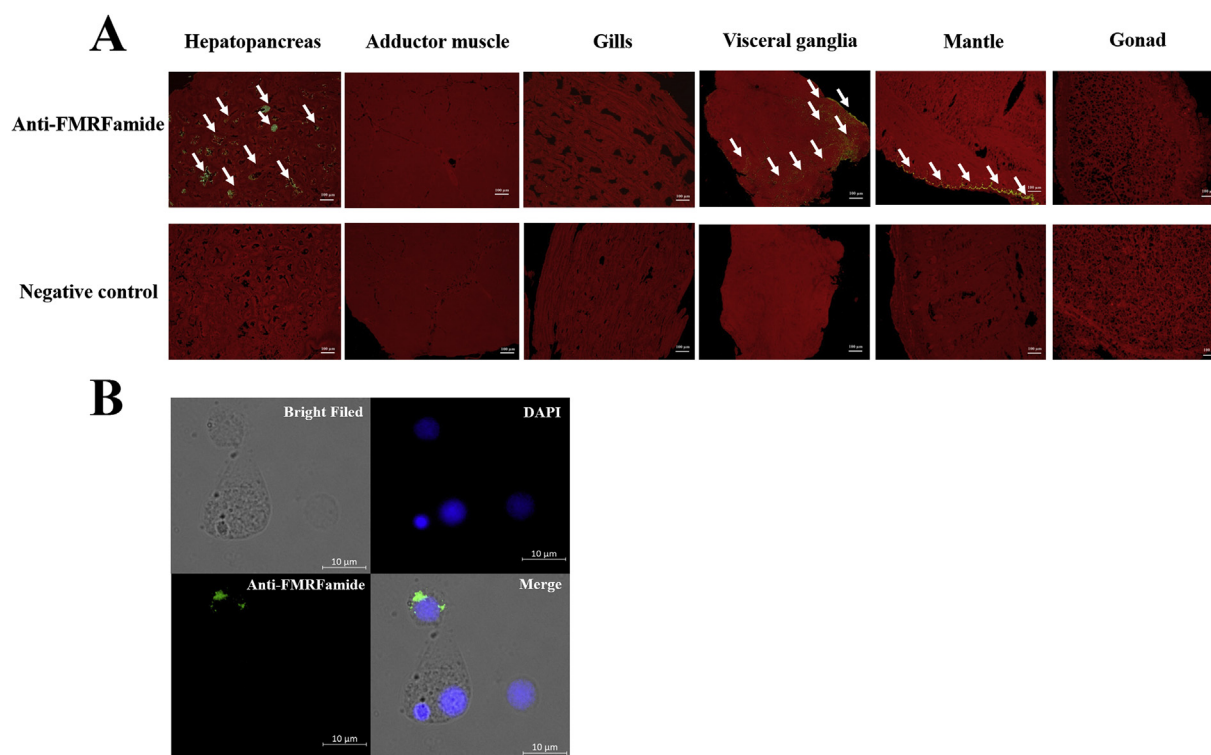


Fig. 4. The distribution of FMRFamide peptide in different tissues of oyster *C. gigas*. (A) Immunohistochemistry analysis of the distribution of FMRFamide in hepatopancreas, adductor muscle, gills, visceral ganglia, mantle and gonad by fluorescence microscope. The red background was stained by Evan Blue dye which could label the cell membrane. The positive signal of FMRFamide was stained with green which was marked by white arrow. The tissues incubated without primary antibody were set as negative control. (B) The cellular distribution of FMRFamide in oyster hemocytes detected by fluorescence microscope. The nucleus of hemocytes was stained with blue (DAPI). The positive signal of FMRFamide was stained with green.

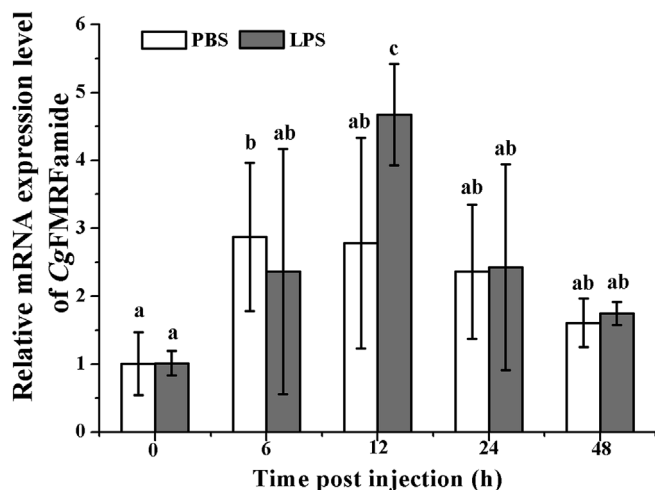


Fig. 5. Temporal mRNA expression of CgFMRFamide in oyster hemocytes after LPS stimulation. Data were represented as the ratio of CgFMRFamide mRNA level to that of 0 h and normalized to that of CgEF. Vertical bars represented the mean $\pm$ S.D. (N = 3) and the significant letters (a, b, etc) indicated significant differences ($p < 0.05$, ANOVA).

phyla of Mollusca, Annelida and Arthropoda. The FMRFamide precursor peptide in *C. gigas* fell into the branch of Bivalvia in phylum of Mollusca, which indicated that CgFMRFamide shared high similarity with the identified FMRFamides in other mollusks.

The gene expression pattern would be a vital guidance to explore the physiological function of CgFMRFamide. As an important component in nervous system of mollusks [29], the mRNA transcript of CgFMRFamide was highest expressed in the visceral ganglia. Compared

with other tissues, CgFMRFamide was also higher expressed in the hepatopancreas of endocrine system and hemocytes of immune system. The mRNA expression pattern of CgFMRFamide was further conformed to the distribution of FMRFamide peptide in different tissues by immunohistochemistry assay. In other mollusks, such as Bivalvia *Macrallista nimbosa* [16], Gastropoda *Helix aspersa* [30] and Cephalopoda *Octopus vulgaris* [31], the FMRFamide peptide was also reported to be mainly concentrated in hemolymph, digestive tracts and nervous system. The wide distribution of FMRFamide in NEI system (visceral ganglia, hepatopancreas and hemocytes) of oyster indicated its important role in homeostasis. Besides, the immunohistochemistry assay revealed that FMRFamide was also expressed in the margin of mantle, indicating that FMRFamide might participate in contracting of mantle muscles, which had been reported in *Dreissena polymorpha* [14]. As the mantle of bivalves is a candidate tissue always invaded by pathogenic organisms [32], FMRFamide existed in the mantle might also participate with immune defense process, such as promoting the expression of immune effectors (CgIL17-5) and apoptosis-related genes (CgCaspase-1 and CgCaspase-3). Interestingly, FMRFamide was also identified in the cytoplasm of oyster hemocytes by immunocytochemistry assays. It was the first report to prove that FMRFamide was produced not only by the nervous/endocrine system, but also synthesized and processed in oyster immunocytes. In general, neuropeptides are respectively released into the hemolymph or synaptic cleft to act as hormones or neurotransmitter by regulating target cells through its receptors [33]. In oyster *C. gigas*, there are totally twenty-eight genes annotated as FMRFamide receptor in the genome, and some of them are highly expressed in the hemocytes according to the transcriptome [34], indicating that oyster hemocytes might be the vital target cells of FMRFamide. It could be speculated that FMRFamide might be synthesized and processed in the visceral ganglia, hepatopancreas and hemocytes, and then released into hemolymph by autocrine/paracrine signaling pathway to regulate the physical

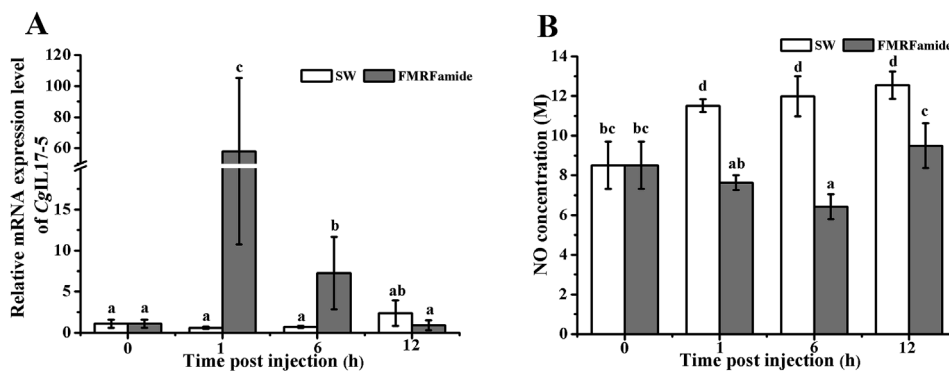


Fig. 6. Temporal mRNA expression of pro-inflammatory cytokine CgIL17-5 in oyster hemocytes (A) and dynamic change of the NO concentration in oyster hemolymph supernatant (B) after *in vivo* stimulation with FMRFamide. Data were represented as the ratio of CgIL17-5 mRNA level to that of 0 h and normalized to that of CgEF. Vertical bars represented the mean $\pm$ S.D. (N = 3) and the significant letters (a, b, etc) indicated extremely significant differences ($p < 0.01$, ANOVA).

functions of hemocytes.

In invertebrates, hemocytes are considered as the immune-related cells playing vital roles in host defense reactions [35]. An increasing number of researches suggested that immunocytes could *de novo* synthesize neuropeptides to regulate immune response by autocrine signaling pathway [1,36,37]. In the present study, FMRFamide was found to be expressed in the hemocytes and its mRNA could be significantly up-regulated at the mid-stage of immune response (12 h after LPS stimulation), which indicated that FMRFamide might be involved in the immune defense. A similar result was also reported in *Biomphalaria* that FMRFamide increased in ganglia after pathogenic *Schistosoma* infection [38].

To further illuminate the possible role of FMRFamide in the immune system of oyster, the dynamically changes of the immune-related molecules were investigated after FMRFamide injection *in vivo*. It has been reported that neuropeptide could regulate the expression of cytokine with versatile effects [39–41]. For example, the pro-inflammatory neuropeptides substance P and K could induce the release of cytokines such as interleukin-1, tumor necrosis factor- α and interleukin-6 to participate in the inflammatory response for eliminating pathogens [39,42]. While some anti-inflammatory neuropeptides, such as vasoactive intestinal peptide and α -MSH, could inhibit the release the inflammatory cytokines to avoid excessive immune reaction [40]. In the present study, FMRFamide was found to dramatically induce the expression of inflammatory cytokines CgIL17-5 (58.1-fold compared with that of in seawater group, $p < 0.01$) in hemocytes at early stage from one hour to six hours, and then, the expression of CgIL17-5 was recovered to normal level at 12 h after FMRFamide stimulation. Given that CgFMRFamide mRNA could be significantly up-regulated at the mid-stage of immune response, it is suggested that FMRFamide could be induced in the mid-stage of immune defense to short-lastingly promote the production of pro-inflammatory cytokines in order to effectively eliminate pathogens, and recovered at the late-stage of immune response to avoid excessive immune reaction. The immune function of FMRFamide was further confirmed to rapidly induce the expression of

apoptosis-related CgCaspase-1 and CgCaspase-3. In mollusks, apoptosis of the immune cells was thought to dampen pathogen spread and protect the integrity of surrounding tissues [43]. It has been reported that IL17 could induce cell apoptosis by activating pro-apoptosis factors in mammals, and inhibit the mRNA expression of apoptosis inhibitor genes in oysters [44,45]. Therefore, it was suggested that FMRFamide could induce the apoptosis of oyster hemocytes through up-regulated pro-inflammatory cytokines for effectively clearing the infected or exhausted cells, and then maintain homeostasis during the immune response. Different from the present results, FMRFamide was reported to protect cell against apoptosis in *Helix lucorum*. There are two possible explanations for those different effects of FMRFamide on cell apoptosis. Firstly, FMRFamide might display different effects on different cell populations. The anti-apoptosis effect of FMRFamide was witnessed in the digestive cells of *H. lucorum* [15]. However, the induced apoptosis effect of FMRFamide was detected in oyster hemocytes in the present study. On the other hand, the effects of FMRFamide on cell apoptosis was also time-dependent. In the present study, the oysters received only an injection with FMRFamide, while FMRFamide injections were repeated four times with an interval of 24 h in *H. lucorum*, which might lead to an adaption of snails to protected cell against apoptosis in *H. lucorum*. Therefore, FMRFamide not induced cell apoptosis, but protected cell against apoptosis in *H. lucorum*.

NO is another immune effector, which has been considered to alter the redox status of hemolymph and influence hemocytes apoptosis in the mollusks [46,47]. In the present study, a continuous down-regulation of NO concentration was detected in the hemolymph supernatant of oysters after injection with FMRFamide for 1–12 h, indicating that the production of NO could be inhibited by FMRFamide. The similar result had been reported in mammalian that the neuropeptide NPFF, a FMRFamide-like substance, could inhibit the release of NO to exert contractile activity in the mouse distal colon [48]. Besides, according to the result that NO displayed a suppression effect on apoptosis of hemocytes in scallops [49], the inhibited production of NO by FMRFamide might also contribute to the increased expression of apoptosis-

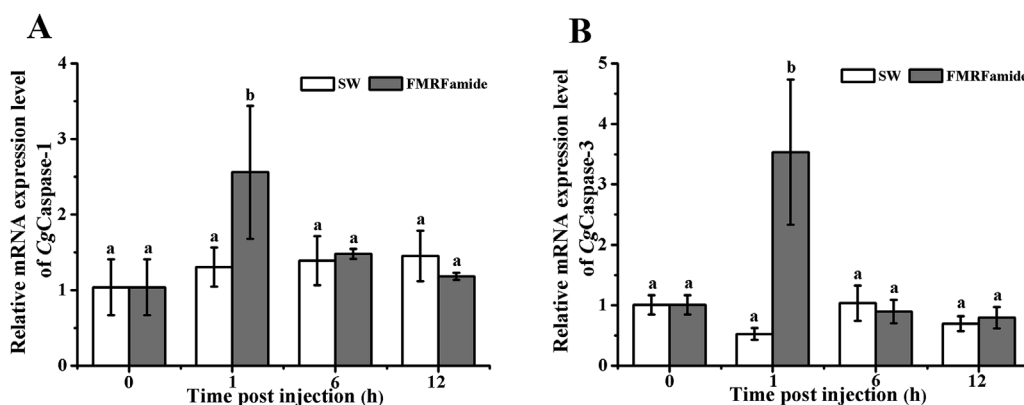


Fig. 7. Temporal mRNA expressions of CgCaspase-1 (A) and CgCaspase-3 (B) after *in vivo* stimulation with FMRFamide. Data were represented as the ratio of CgCaspase-1 and CgCaspase-3 mRNA level to that of 0 h and normalized to that of CgEF, respectively. Vertical bars represented the mean $\pm$ S.D. (N = 3) and the significant letters (a, b, etc) indicated significant differences ($p < 0.01$, ANOVA).

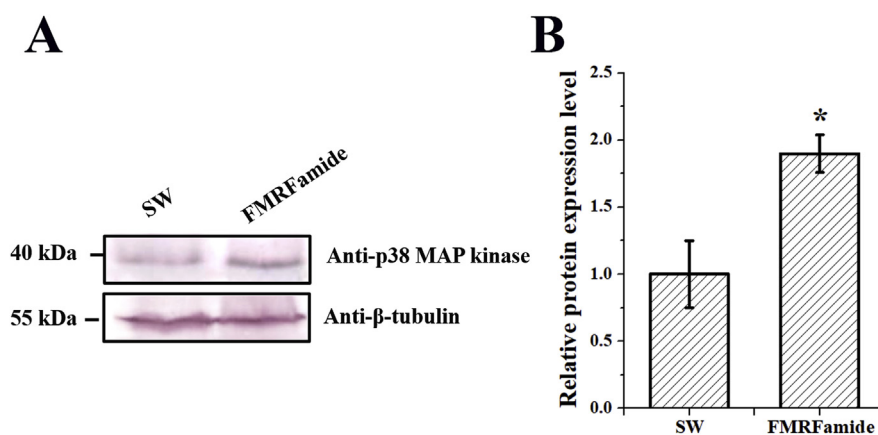


Fig. 8. Effect of FMRFamide on the phosphorylation of p38 MAP kinase in oyster hemocytes. (A) The p38 MAP kinase in oyster hemocytes was detected with phospho-p38 MAP kinase antibody after FMRFamide/seawater (SW) stimulation for one hour, and the β-tubulin detected with β-tubulin antibody was used as control. (B) Statistical analysis the ratio of phospho-p38 MAP kinase/tubulin according to the grey value by ImageJ software. Vertical bars represented the mean ± S.D. and asterisk indicated significant differences ($p < 0.05$, t -test).

related gene in hemocytes of oysters.

P38 MAP kinase is crucial for the production of inflammatory cytokine and induction of cell apoptosis in vertebrates [50,51]. In the present study, the expression of CgIL17-5 and the phosphorylation level of p38 MAP kinase in hemocytes both increased significantly after FMRFamide stimulation, indicating that the p38 MAP kinase signal pathway was activated by FMRFamide, which was consistent with the results reported in nervous system of *Aplysia* [12]. In addition, p38 MAP kinase was also reported to influence on the apoptosis of immunocytes and played a key mediator in glucocorticoid-induced apoptosis of lymphoid cells [52]. Therefore, the increased p38 MAP kinase in oyster hemocytes might mediate the effects of FMRFamide on the expression of pro-inflammatory cytokines and apoptosis of oyster hemocytes.

In conclusion, a unique FMRFamide precursor gene was identified in oyster *C. gigas*. The mRNA of CgFMRFamide precursor and peptide of FMRFamide were both mainly concentrated in the visceral ganglia, hepatopancreas and hemocytes, and the expression of CgFMRFamide in hemocytes was significantly increased in response to LPS stimulation. FMRFamide could up-regulate the expressions of pro-inflammatory cytokines CgIL17-5 and apoptosis-related genes CgCaspase-1 and CgCaspase-3, but inhibit the production of NO. The immune regulatory effect of FMRFamide could be mediated by the increased phosphorylation of p38 MAP kinase signal. These results collectively demonstrated that the conserved FMRFamide could not only respond to immune stimulation, but also regulate the expressions of immune effectors and apoptosis-related genes to mediate p38 MAP kinase pathway, and eventually maintain the homeostasis in oysters.

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