**CgA1AR-1 acts as an alpha-1 adrenergic receptor in oyster
Crassostrea gigas mediating both cellular and humoral
immune response**

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Abstract

We have now cloned an alpha-1 adrenergic receptor (A1AR) from the cDNA library of oyster *Crassostrea gigas*, designating as CgA1AR-1. The full length of CgA1AR-1 was 1149 bp and it encodes a protein of 382 amino acids containing a 7 transmembrane domain, whose putative topology was similar to the A1AR in higher organisms and shared similarity of 19% with mammalian A1ARs according to the phylogenetic analysis. After cell transfection of CgA1AR-1 into HEK293T cells and the incubation with its specific agonist norepinephrine (NE), the concentration of second messenger Ca^{2+} increased significantly ($p < 0.05$). But, this increasing of Ca^{2+} could be inhibited by adding A1AR antagonist DOX. Tissue distribution assays using

qRT-PCR suggested that CgA1AR-1 mRNA was ubiquitously expressed in all the major tissues of oyster. LPS stimulation could induce the up-regulation of CgA1AR-1 mRNA in haemocytes from 12 h to 24 h post stimulation. Moreover, the blocking CgA1AR-1 by DOX before LPS stimulation affected the mRNA expression of oyster TNF (CGI_10005109 and CGI_10006440) in haemocytes, resulting in the rise of haemocyte phagocytic rate and apoptosis index. In addition to cellular immunity, CgA1AR-1 was also involved in humoral immunity of oyster. Inhibition of CgA1AR-1 with DOX could repress the up-regulate of LZY and SOD activities caused by LPS stimulation. These results suggested that CgA1AR-1 acted as a α -1 adrenergic receptor in cetacholaminergic neuroendocrine-immune network mediating both cellular and humoral immune response.

Key words: *Crassostrea gigas*; Norepinephrine; Alpha-1 adrenergic receptor; TNF pathway; Immune regulation

1. Introduction

Neuroendocrine hormones and neurotransmitters have been proved to play important role in the maintenance of homeostasis under environmental stress [1]. Norepinephrine (NE), together with the other two catecholamines (CA) adrenaline and dopamine, is the very first neurotransmitter responsive to stress, causing the up-regulation of immunity modulation [2, 3]. Complicated catecholaminergic neuroendocrine system has been found in all vertebrates, while the recent reports evidenced that intact catecholaminergic system also existed in molluscs [4]. For instance, CA, CA receptors and CA synthesis-related key enzymes have been identified in marine shellfishes [5-7].

Although CA is known to play essential role in feeding [8], respiration [9], development [10], locomotion [11] and reproduction [12] of invertebrates, its role in the immune regulation tops them all. For example, when *Litopenaeus vannamei* [13] and *Macrobrachium rosenbergii* [14] were infected by bacteria, NE injection could inhibit the immune responses. Lacoste et al reported for the first time in molluscs that CA was related to the immune regulation [15-18]. They revealed that the phagocytosis of haemocyte and ROS production could be modulated by NE in oyster *C. gigas* [13, 18]. Molluscs have an open circulatory system and no particular immune organs; the immunomodulation is mediated mainly by the mobile immune cells (haemocytes), which enables the host to maintain immune surveillance during or after stressful events that threaten the animal's survival [19]. Secretion of NE occurs in response to stress in molluscs, which has been shown to modulate the immunomodulation of

oyster haemocyte [20]. For instance, NE stimulation could induce transcriptional upregulation of hsp70 in molluscan haemocytes, which was thought to inhibit the apoptotic process [21]. Also, NE can inhibit the production of TNF-alpha (via beta-adrenergic receptors) in haemocytes under LPS stimulation [22]. Similar to the immune cells in vertebrates, molluscan haemocytes express receptors for many hormones or neurotransmitters [4, 23, 24], and the immune responses are regulated by neurotransmitters or hormones mainly through the activation of these receptors [25].

NE binds to its specific receptors, adrenergic receptors (ARs), to accomplish its functions. ARs belong GPCRs super family[1], and are divided into two major classes, α and β [26]. The binding of NE to α - and β -ARs can induce the fluctuation of intracellular Ca^{2+} and cAMP [27]. The α -1 adrenergic receptor (A1AR) couples to G_q protein and increases the fluxes of intracellular Ca^{2+} , while α -2 adrenergic receptor (A2AR) binds to G_i protein to down-regulate cAMP concentration. On the other hand, β adrenergic receptor (BAR) prefers to bind to G_s protein, causing the up-regulation of cAMP [4]. Activation of different subtypes of ARs results in diverse immune regulation patterns. As for A1ARs, they are expressed on both neuronal and immune cells, modulating the neurotransmission and a series of neurodegenerative disorders [28]. It has been reported that the expression of A1AR would increase on mononuclear immune cells under chronic inflammation [26]. However, little is known about the adrenergic immunomodulation in molluscs with few genes, especially receptors, identified and validated.

Living in intertidal and estuarine regions, seashells like oysters have to deal with

the severe dynamically fluctuating environment [29]. NE and ARs have previously been reported to be existed in scallop *C. farreri* and played significant roles in the immunomodulation as well as adaptation to the environment [4]. Taking into account the significant physiological and immunological functions of NE and ARs, the investigation about ARs in molluscs will shed light on a better study of the adrenergic neuroendocrine-immune network as well as the immunomodulation mechanism in marine invertebrates. The aims of this research are to (1) amplify the receptor for NE from oyster *C. gigas* (designated as CgA1AR-1), (2) study the fluctuation of second messengers Ca^{2+} and cAMP after the activation of CgA1AR-1, (3) examine the change of phagocytic rate and apoptosis index of oyster haemocytes, as well as the expression of CgTNFs and the activity of immune-related enzymes after the activation of CgA1AR-1 to understand its possible function in the immune response.

2. Materials and Methods

2.1. Oysters, antagonist incubation, LPS stimulation and sample collection

The adult pacific oyster *C. gigas* with the average shell length of about 15.0 cm was purchased from a local breeding base in Qsingtiao, China. We drilled a small hole on each oyster's shell near the adductor muscle and maintain the oysters in clean seawater at 20 °C for at least two weeks, and feed the oysters with algae powder and replace the water in the tanks every other day.

Doxazosin mesylate (DOX, TOCRIS), idazoxan yohimbine (IDA, TOCRIS) and propranolol (PRO, TOCRIS) [30-32] were A1AR, A2AR and BAR antagonist,

111 respectively. In the antagonist incubation assays for cultured HEK293T cells, 100 μL
112 of antagonist (1.0 mmol L^{-1}) was added to the cell medium for an incubation of 1 h
113 before LPS stimulation. Similarly, in the incubation experiments on adult oyster, 100
114 μL of antagonist with the same concentration was injected into the adductor muscle
115 near the blood sinus, and at 12 h after DOX injection, LPS was injected as described
116 below.

117 LPS was injected into the oyster to activate the immune responses. Here we
118 injected 100 μL of LPS (0.5 mg mL^{-1}) from *Escherichia coli* 0111:B4 (Sigma)
119 through the tiny hole in the LPS groups, while oysters in the PBS group were treated
120 with PBS (pH 7.4). Fifteen were sampled at each time point after LPS stimulation as
121 one replicate, and three replicates were taken for each test. The oysters in the blank
122 group were collected at 0 h without any treatment.

123 We draw the hemolymph with a thin syringe from the blood sinus and
124 centrifuged them at 800 g to gather the haemocytes and serum. In order to obtain
125 single-cell suspension for the primary cell culture, the acid citrate-dextrose
126 anticoagulant reagent (22 g/L Sodium Citrate, 8 g/L citric acid, 24.5 g/L glucose, pH
127 7.4) at the ratio of 8:1 was used. Hemolymph from five oysters was employed as one
128 duplicate and three parallel duplicates were performed for each time point. Seven
129 major tissues including gonad, gill, mantle, kidney, haemocytes, adductor muscle and
130 hepatopancreas were collected for tissue distribution analysis. 1 mL Trizol reagent
131 (Invitrogen, USA) was added to each tube containing haemocytes and tissues for
132 RNA extraction. Haemocytes, tissues and serum were all frozen immediately with

liquid nitrogen after sampling and stored at -80 °C.

2.2. RNA extraction, cDNA synthesis and qRT-PCR analysis of CgA1AR-1 and TNF mRNA expression

We extracted total RNA from haemocytes and tissues using Trizol reagent (Invitrogen, USA). The cDNA library was constructed using Promega M-MLV kit, and diluted to 1:40 for the subsequent experiments.

We employed qRT-PCR and $2^{-\Delta\Delta C_t}$ method to determine and calculate the mRNA expression level of CgA1AR-1 and two oyster's TNF (CGI_10005109 and CGI_10006440) in haemocytes after LPS stimulation according to Zhang *et al* [33]. All the primers used were listed in Table 1 and the fragment (168 bp) of oyster elongation factor (CgEF, CGI_10012474) was detected as endogenous control. Six duplicates were tested for each sample.

2.3. Gene cloning and sequence analysis

The ORF of CgA1AR-1 (No. **EKC29764.1**), which was homologous to vertebrate A1AR, was obtained and analyzed. The Expert Protein Analysis System was applied in the amino acid analysis. The protein domain was predicted with SMART software. Multiple alignment of the CgA1AR-1 with adrenergic receptors from other species was employed by the ClustalW software. Phylogenetic tree of A1ARs was built by MEGA software using neighbor-joining (NJ) algorithm.

2.4. Cell transfection, drug treatment and second messenger determination

HEK293T cells were cultured as described by Liu et al [24]. The full length sequence of CgA1AR-1 was amplified with corresponding primers and sub-cloned into the pEASY-M₁ expression vector (TransGene), designating as pEASY-M₁-CgA1AR-1 plasmid. When the cell coverage ratio reached 60~80 % in the wells, transfect the recombinant plasmid with Lipofectamine LTX and PlusTM Reagent (Invitrogen). After being cultured for 24 hours, the cells were digested and collected for the second messenger determination.

For the stimulation assay, 500 μ L cell of resuspension (about 10^4 cells) of HEK293T with pEASY-M₁-CgA1AR-1 was added into the 24-well plate with 50 μ L NE (of 10^{-4} mol L⁻¹, Sigma Aldrich) [4], which was designed as CgA1AR-1 group. In the CgA1AR-1+DOX group, 50 μ L of 10.0 μ mol L⁻¹ DOX was firstly added, incubating for 6 h, and then add 50 μ L of NE to the cells. Similarly, specific antagonists IDA and PRO for A2AR and BAR were also added to the wells of transfected HEK293T cells before the addition of NE, named as CgA1AR-1+IDA and CgA1AR-1+PRO group, respectively. Cells without CgA1AR-1 gene transfection nor NE stimulation were collected as the blank group, while cells transfected with CgA1AR-1 but not incubated with NE was designated as the control group. The blank group referred to cells without transfection or stimulation, and the control group referred to cells with cell transfection but not stimulated by NE. After NE treatment for 1 h, HEK293T cells were digested and harvested. All incubation assays were carried out by 3 replicates.

Fluo-3 AM fluorescent probe (Beyotime, 1.0 $\mu\text{mol L}^{-1}$) was employed in the examination of intracellular Ca^{2+} , and the cAMP Direct Immunoassay Kit (ab65355, Abcam, Cambridge, UK) was used to measure cAMP concentration as illustrated [34]. Three duplicates were examined for each test.

2.5. Determination of the phagocytic rate and apoptosis index of oyster haemocytes

The phagocytic rate of haemocytes were measured according to Liu *et al* [34]. Firstly, we resuspended haemocytes with seawater and adjusted the cell count to $1.0 \times 10^6 \text{ cell mL}^{-1}$, and then incubate every 500 μL haemocytes with 5 μL *Vibrio anguillarum* dyed by FITC ($1.0 \times 10^9 \text{ CFU mL}^{-1}$) for 1 h. The haemocytes were centrifuged to elute the extra bacteria. Then, the FITC intensity was detected by flow cytometry and the phagocytic rate was calculated as Number of Phagocytic Cells with Engulfed Bacteria/ Number of Phagocytes. The apoptosis index of haemocytes was measured using Annexin V-FITC Apoptosis Detection Kit (KeyGEN, China). Resuspend haemocytes and incubate them with Annexin V-FITC for 10 min. Centrifuge at 1000 g for 5 min, followed by resuspension of haemocytes and incubation with Propidium Iodide at room temperature in darkness. The apoptosis index was calculated as Number of Apoptotic Cells/ Number of Haemocytes. Three parallel replicates were performed in each phagocytosis and apoptosis assay.

2.6. Activity measurement of immune-related enzymes

In the present study, we measured the activities of SOD (superoxide dismutase),

LYZ (lysozyme) and CAT (catalase), which are the important enzymes in the immune regulation using the kit from Nanjing, Jiancheng (A001-1, A050-1 and A007). Three parallel replicates were performed in each enzyme-activity assay.

2.7. Statistical analysis

All values obtained were listed as means $\pm$ SD and analyzed by one-way ANOVA and a Student-Newman-Keus (S-N-K) comparison. Letters (a/b/c, etc.) were marked to illustrate the statistical significance ($p < 0.05$).

3. Results

3.1. The molecular characters of CgA1AR-1 and phylogenic relationship

The nucleotide sequence of CgA1AR-1 was 1149 bp (No. **EKC29764.1** in GenBank), and the polypeptide sequence contained 382 amino acids with the molecular mass predicted as 43.18 KDa. The theoretical isoelectric point of CgA1AR-1 was 8.82 and a seven transmembrane region (from Leu20 to Ser366) was revealed by SMART analysis.

CgA1AR-1 shared 19.0 % similarity *Homo sapiens* A1AR, and 19.6 % similarity with *Bos Taurus* A1AR. Five conserved cysteine residues (Cys92, Cys100, Cys168, Cys197 and Cys321) were identified by the alignment of CgA1AR-1 with other two A1ARs from vertebrates. PROSITE website predicted that there was an conserved disulfide bridge from Cys91 to Cys168 (Fig. 1).

A1ARs from oyster and other 9 animals were obtained from NCBI website to build a phylogeny tree by MEGA 5 (Fig. 2). These A1ARs were positioned separately in two major groups, ARs and BRs. The separation between different receptors was in line with their subtypes. According to the phylogenic tree, CgA1AR-1 was closely matched with A1ARs previously identified in vertebrates.

3.2. Change of Ca^{2+} and cAMP concentrations after NE treatment

Change of intracellular second messengers Ca^{2+} and cAMP after CgA1AR-1 gene transfection and NE incubation was tested to explore the binding activity of CgA1AR-1 and its possible subtype (Fig. 3A). The results showed that Ca^{2+} level in the CgA1AR-1 group increased dramatically ($p < 0.05$) after NE stimulation, which was 1.77-fold compared with that in the control group (Fig. 3B). Dramatic down-regulation of Ca^{2+} occurred in the CgA1AR-1+DOX group at 1 h after NE stimulation. There was no significant change in the CgA1AR-1+IDA and CgA1AR-1+PRO groups. On the contrary, the concentration of intracellular cAMP remained unchanged between the experimental and control groups ($p > 0.05$) (Fig. 4).

3.3. The expression of CgA1AR-1 in various tissues and in haemocytes after LPS stimulation

We carried out qRT-PCR to explore the expression of CgA1AR-1 in all kinds of oyster tissues and its response to LPS stimulation. The mRNA transcripts of CgA1AR-1 were determined in all seven tissues including haemocytes, mantle,

kidney, gill, gonad, hepatopancreas and adductor muscle. CgA1AR-1 was highly expressed in hepatopancreas, which was 12.69-fold compared with that in gonad ($p < 0.05$). The expressions of CgA1AR-1 were also quite high in adductor muscle and kidney, which were about 10.41-fold and 2.95-fold ($p < 0.05$) (Fig. 5).

LPS stimulation could result in the increase of CgA1AR-1 mRNA expression in haemocytes from 12 h to 24 h (5.72-fold, $p < 0.05$). At 96 h post stimulation, the expression level decreased to normal level at 96 h compared to PBS group (0.77-fold, $p > 0.05$) (Fig. 6).

3.4. The change of TNF mRNA expression after the inhibition of CgA1AR-1 and LPS stimulation

The mRNA expression levels of two oyster TNF genes (CGI_10005109 and CGI_10006440) in haemocytes after DOX incubation and LPS stimulation were determined (Fig. 7). After the treatments with CgA1AR-1 antagonist DOX and LPS, the expressions of both TNF genes were up-regulated significantly from 3 h to 12 h ($p < 0.05$). The expression level of CGI_10005109 was much lower at 6 h and 12 h in the LPS+DOX group of that in PBS group ($p < 0.05$), while CGI_10006440 expression was higher at 3 h in the antagonist incubation group ($p < 0.05$).

3.5. Phagocytic rate and apoptosis index of oyster haemocyte after CgA1AR-1 antagonist incubation and LPS stimulation

In order to explore the possible modulation of cellular immunity mediated by

CgA1AR-1, the phagocytic rate and apoptosis index of oyster haemocyte were determined by flow cytometry after 12 h of CgA1AR-1 antagonist incubation and 12 h of LPS stimulation (Fig. 8 and Fig. 9). The phagocytic rate and apoptosis index both increased significantly after LPS stimulation ($p < 0.05$). After an incubation with CgA1AR-1 specific antagonist DOX, the phagocytic rate increased significantly from 18.9 % in the LPS group to 21.7 % in the LPS+DOX group ($p < 0.05$) (Fig. 8B). Similarly, apoptosis index of oyster haemocyte increased dramatically from 12.8 % in the LPS group to 16.1 % in the LPS+DOX group ($p < 0.05$) (Fig. 8B).

3.6. The effects of A1AR antagonist on the activities of immune effectors

In order to explore the possible modulation of humoral immunity mediated by CgA1AR-1, the activities of SOD, CAT and LYZ, three key immune enzymes, were determined. The activities of all three enzymes were significantly up-regulated (6 h post LPS stimulation) ($p < 0.05$) (Fig. 10). Moreover, the activities of SOD and LYZ were significantly higher after DOX incubation (Fig. 10A and Fig. 10B), while the activities of CAT in the antagonist incubation group showed no dramatic difference compared with LPS group (Fig. 10C).

4. Discussion

The clearance of invading pathogens and minimization the damage to the host to maintain homeostasis under environmental stress depends mainly on the neuroendocrine-immune interactions [35]. One of the most typical examples of this in

molluscs is the modulation of catecholaminergic nervous system. For instance, the catecholaminergic network identified in scallop could be activated by pathogen affection and release catecholamines to negatively modulate the immune response [4]. The adrenergic receptors are a family of GPCRs, which are targets of CA, especially NE and EPI. The classification and functional characterization of ARs in vertebrates is quite profound, while little is known about invertebrate ARs.

In the present study, we amplified an alpha-1 subtype receptor taking adrenaline as its binding ligand, naming as CgA1AR-1. Its ORF was of 1149 bp and the polypeptide it encoded consisted of 382 amino acids. The amino acid sequence of CgA1AR-1 shared 19.0 % similarity with that of *Homo sapiens* A1AR, and 19.6 % similarity with that of *Bos Taurus* A1AR. Five conserved cysteine residues were identified by the alignment analysis in CgA1AR-1, and the conserved Cys91 and Cys168 were predicted to form an important disulfide bridge, which might be critical for its binding activity [36]. Phylogeny analysis of CgA1AR-1 and other ARs from different animals was conducted to explore the possible subtype of CgA1AR-1. According to the phylogeny tree, CgA1AR-1 was closely matched with A1ARs identified in vertebrates, and it might be crucial for locomotion, cytokine production and the phagocytosis of the immune cells [17]. Moreover, The GPCRs share a common topographical motif consisting of seven putative transmembrane regions joined by intracellular and extracellular loops. Studies with chimeric α_2/β_2 AR (5) and muscarinic cholinergic receptors (6) have shown that the putative third intracellular loop contains the structural determinants for receptor coupling to specific G proteins

[37, 38]. Also, it was reported that amino acid residues in the N- and C-terminal portions of the third loop play a prominent role in α_1 AR coupling to phospholipase C (PLC). Thus, the Cys residues near the N-terminal might be a conserved region and crucial for the binding activity of A1ARs.

The up-stream signals from neuroendocrine system were relayed into the immune system through the activation of neurotransmitter/hormone receptors as well as the fluctuation of intracellular second messengers [39]. To understand the function of CgA1AR-1 and its possible subtype, we measured the concentrations of Ca^{2+} and cAMP after transfection and NE treatment. The concentration of intracellular Ca^{2+} was significantly increased ($p < 0.05$) after CgA1AR-1 transfection and NE treatment, which was 1.77-fold of that in the control group. However, the cAMP concentration showed no significant change after NE treatment. It has been reported that α -1 adrenergic receptor (A1AR) couples to G_q protein, causing the accumulation Ca^{2+} , while α -2 adrenergic receptor (A2AR) prefer to bind to G_i protein to decrease cAMP concentration. In addition, intracellular cAMP arises when β adrenergic receptor (BAR) is activated by coupling to G_s [4]. The change of intracellular second messengers in the present study implied that CgA1AR might be a member of A1ARs. Furthermore, incubation of A1AR selective antagonist DOX caused a dramatic decrease of intracellular Ca^{2+} concentration, while the concentration of intracellular Ca^{2+} in the CgA1AR-1+IDA (A2AR selective antagonist) and CgA1AR-1+PRO (BAR non-selective antagonist) groups did not change significantly ($p > 0.05$), further ascertaining that CgA1AR-1 belonged to A1AR family. As far as we know, the

331 signaling transduction pattern of ARs between vertebrates and invertebrates is quite
332 conserved. Collectively, CgA1AR-1 should be seen as one of the A1ARs in oyster,
333 which rely on the concentration change of Ca^{2+} instead of cAMP to transfer signals
334 into downstream pathways.

335 qRT-PCR was employed in the tissue distribution assay of CgA1AR-1 for a
336 better exploration of its physiological role in oyster. In the present study, CgA1AR-1
337 was expressed in every tissue tested, suggesting that it should be an indispensable
338 molecules in oyster. It is said that A1AR is responsible for movement of smooth
339 muscle [28], the detection of CgA1AR-1 in adductor muscle indicated that it affected
340 muscle movement. Besides, the high expression of CgA1AR-1 in hepatopancreas, the
341 most important immune tissue in molluscs [40, 41], indicated that it could be the
342 hub-gene in the immune-related gene network. Moreover, the mRNA transcripts were
343 also detected in gill and mantle, which are seen as the first defense line for the
344 filter-feeding molluscs as oyster.

345 The circling haemocytes mediates a complex process in which immune and
346 neuroendocrine responses are intermixed to help the lower forms of life successfully
347 live and survive in environments full of potentially agents and substances [42, 43]. As
348 a key neurotransmitter, NE could modulate the immune response mainly through ARs.
349 Here in this research, the temporal change of mRNA expression level of CgA1AR-1
350 in haemocytes was determined to illustrate its possible role in the immunomodulation.
351 After LPS stimulation, CgA1AR-1 mRNA overexpressed at 12 h and its expression
352 peaked at 24 h (5.72-fold, $p < 0.05$). Since the release of cytokines would increase to

enhance the immune responses once the pathogens were recognized by the pathogen recognition receptors (PRRs) [23]. In the present research, the recognition of LPS by PRRs was found to induce the expression of CgA1AR-1, which might regulate the early immune regulation to accelerate the elimination of pathogens. However, the sustained high-level immune response would in turn cause immune injury. In order to avoid killing normal cells and maintain homeostasis, more receptors with inhibitory effect such as α -adrenergic receptor [4] will be expressed on the haemocytes surface to inhibit the response level [44]. The fall of CgA1AR-1 mRNA expression level at 96 h post LPS treatment should be the indication of immune response switch from high to the normal level. These results collectively suggested that CgA1AR-1 was an indispensable element in the catecholaminergic immune regulation in oyster, which meant a world for the pathogen clearance and homeostasis maintenance.

The interaction between immune and nervous system is vital for the regulation of innate immune response and inflammation control [45, 46], in which cytokines such as TNF, IL and IFN play indispensable roles. In the current research, the expression of two oyster TNFs (CGI_10005109 and CGI_10006440), as well as some immunological indexes including haemocyte phagocytic rate, apoptosis index and the activities of three immune effectors (SOD, LYZ and CAT), were determined to further understand the immunomodulation mediated by CgA1AR-1. The expression of CGI_10005109 and CGI_10006440 was up-regulated after LPS treatment, and the up-regulation of CGI_10006440 was further enhanced after CgA1AR-1 was inhibited by its antagonist DOX. LPS stimulation could activate the neuroendocrine system to

375 release neurotransmitters including NE, enkephalin (ENK) [24, 47] and ACh [48] to
376 regulate the immune responses through cytokines, such as IL-17 [49], IFN- γ [50] and
377 TNF- α [51] in mollusc. The change of TNF expression after blockage of CgA1AR-1
378 suggested that CgA1AR-1 could regulate TNF expression as an immunomodulation
379 manner of NE in oyster. Besides, both phagocytic rate and apoptosis index increased
380 significantly after LPS stimulation ($p < 0.05$), and they were further induced
381 significantly after the incubation of CgA1AR-1 specific antagonist, indicating that
382 CgA1AR-1 was responsible for the cellular regulation of oysters against infection.
383 Moreover, the activities of SOD and LYZ in the LPS+DOX groups were significantly
384 higher compared with LPS groups, while the activities of CAT showed no dramatic
385 difference between the above two groups. Previous studies demonstrated that acute
386 stress such as pathogen invasion could suppress the immune response level in
387 molluscs. This suppression of the immune function is caused, at least in part, by the
388 release of NE [52]. Molluscan haemocytes contain receptors for NE, and NE has
389 negative effects on haemocyte function [4, 53-55]. For example, injection of NE
390 resulted in decreased bacterial clearance and increased mortality in oysters challenged
391 with a bacterial pathogen [56]. In the present study, the activities of SOD, CAT and
392 LYZ all increased significantly after LPS stimulation, suggesting that the humoral
393 immune response in oyster was activated after LPS injection. Moreover, the activities
394 of SOD and LYZ in the groups which the CgA1AR-1 was blocked by antagonist
395 DOX decreased significantly compared with that in the LPS groups, implying that NE
396 down-regulated the immune response caused by LPS stimulation by the mediation of

its receptor CgA1AR-1, and the blockage of CgA1AR-1 could revert the results.

In conclusion, CgA1AR-1 was identified from oyster *C. gigas*. The expression of CgA1AR-1 mRNA in haemocyte was significantly induced after LPS stimulation. The blockage of CgA1AR-1 using specific antagonist before LPS stimulation affected the expression of oyster TNF, resulting in the increase of haemocyte phagocytic rate, apoptosis index, as well as the activities of the SOD and LYZ. The immune responses induced by LPS challenge could be dramatically inhibited under the regulation of high concentration of NE (10^{-4} mol L⁻¹), and this trend would be reverted after CgA1AR-1 was blocked by its antagonist. These results suggested that A1AR in oyster plays a significant role in catecholaminergic immunomodulation to protect the host from pathogen infection and maintain homeostasis.

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419

420 **Figure legend**

421 Fig.1. Multiple alignment of CgA1AR-1 with A1ARs from model species. The black
422 shadow region indicates positions where all sequences shared the same amino acid
423 residue, while the similar amino acids are shaded in grey. Gaps were marked by
424 dashes to improve the alignment. The asterisks indicate the conserved cysteine
425 residues.

426

427 Fig.2. Consensus neighbor-joining (NJ) phylogeny tree of adrenoceptors from
428 different animals, such as *H. sapiens*, *D. rerio*, and *C. gigas*. The phylogeny tree was
429 constructed by MEGA 4 software.

430

431 Fig.3. Change of intracellular Ca^{2+} in HEK293T cells after AR antagonists incubation
432 and NE treatment. (A) The intracellular Ca^{2+} concentration determined by flow
433 cytometry. (B) The overall concentration change of intracellular Ca^{2+} in HEK293T
434 cells after cell transfection, A1AR, A2AR and BAR antagonists incubation and NE
435 stimulation.

436

437 Fig.4. Change of intracellular cAMP in in HEK293T cells after cell transfection, AR
438 antagonists incubation and NE treatment. Each group value is shown as mean $\pm$ SD,
439 and bars with asterisks are significantly different ($p < 0.05$).

440

Fig.5. Tissue distribution of CgA1AR-1 detected by qRT-PCR. CgA1AR-1 relative mRNA expression level in haemocyte, mantle, kidney, gill, muscle and hepatopancreas was normalized to that of gonad. Each group value is shown as mean $\pm$ SD, and bars with different letters are significantly different ($p < 0.05$).

Fig.6. Temporal expression of CgA1AR-1 mRNA in haemocytes after PBS and LPS stimulation. CgA1AR-1 relative mRNA expression level in the blank group was normalized as 1. Each group value is shown as mean $\pm$ SD, and bars with different letters are significantly different ($p < 0.05$).

Fig.7. Relative expression of two TNF genes in oyster haemocytes after A1AR antagonist DOX incubation and LPS stimulation. CgTNF relative mRNA expression level in the blank group was normalized as 1. Each group value is shown as mean $\pm$ SD, and bars with different letters are significantly different ($p < 0.05$).

Fig.8. Effect of *in vivo* incubation with CgA1AR-1 antagonist and stimulation of LPS on haemocyte phagocytic rate. (A) Determination of haemocyte phagocytic rate by flow cytometry. (B) The overall change of haemocyte phagocytic rate after the incubation of CgA1AR-1 antagonist and LPS stimulation.

463

464 Fig.9. Effect of *in vivo* incubation with CgA1AR-1 antagonist and stimulation of LPS
465 on haemocyte apoptosis index. (A) Determination of haemocyte apoptosis index by
466 flow cytometry. (B) The overall change of haemocyte apoptosis index after the
467 incubation of CgA1AR-1 antagonist and LPS stimulation.

468

469 Fig.10. Activities of three immune-related enzymes after antagonists incubation and
470 LPS stimulation. Each group value is shown as mean $\pm$ SD, and bars with asterisks
471 are significantly different ($p < 0.05$).

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AlAR_Homo_sapiens  MVFLSGNASDSSNCTQSPAPVNIKKALLGVILGGILFGVIGNILVITSVACRRHLSTHYVYVNLAVRDLTSTYLPSSAFTEVLGYWAFGRVFCN 100
AlAR_Bos_taurus   MVFLSGNASDSSNCTHPFPFVNIKKALLGVILGGILFGVIGNILVITSVACRRHLSTHYVYVNLAVRDLTSTYLPSSAFTEVLGYWAFGRVFCN 100
CqAlAR_Crassostrea_gigas  -----MNVSASSVEISLGVHNSKILLGVFTYLVSVITIGNILVITSVACRRHLSTHYVYVNLAVRDLTSTYLPSSAFTEVLGYWAFGRVFCN 92

AlAR_Homo_sapiens  IWAADVLCCTASINGLCIISIDRYEGVSYELRYPTIVTORRGMAALCVNALSLVSTIGELFGWRQEPFEDITICQINEEPGYVLFSAIGSFYLFELAI 200
AlAR_Bos_taurus   IWAADVLCCTASINGLCIISIDRYEGVSYELRYPTIVTORRGMAALCVNALSLVSTIGELFGWRQEPFEDITICQINEEPGYVLFSAIGSFYLFELAI 200
CqAlAR_Crassostrea_gigas  VVWIFFYVGMTFVSFTLCIISIDRYEGVSYELRYPTIVTORRGMAALCVNALSLVSTIGELFGWRQEPFEDITICQINEEPGYVLFSAIGSFYLFELAI 192

AlAR_Homo_sapiens  LVMVCSVVVAKRESGLKSGGLKIDSDSEVILRIIRKNAQVGSSEMASAKIKTHFSVRLKRSRKKTAFTLSVVGCFVLCNLFFFLVMPIGSFFPD 300
AlAR_Bos_taurus   LVMVCSVVVAKRESGLKSGGLKIDSDSEVILRIIRKNAQVGSSESVISAKNKTHFSVRLKRSRKKTAFTLSVVGCFVLCNLFFFLVMPIGSFFPD 300
CqAlAR_Crassostrea_gigas  VVIFGCVTFVTFVVRQRKTAIAENKTNSAATASMASA---SQQMSDINTCQSETENFSSVSVVCSNDASNQLQVFI----- 266

AlAR_Homo_sapiens  FRPSSIVKRIVFWLGYLNSCINPIIIFCSSSEKKAFQNVILRIQCLRRQSSSKHAGYTHFPFSQAVEGQHKDMVRIVGSRETIFYRISKTDGVCENKFF 400
AlAR_Bos_taurus   FRPSSIVKRIAFWLGYLNSCINPIIIFCSSSEKKAFQNVILRIQCLRRQSSSKHTGYTHAPSHVLEGQHKDLVRIVGSRETIFYRISKTDGVCENKIF 400
CqAlAR_Crassostrea_gigas  MNKDSSVMDSS-----SGTSVSEKTDGDKIVMFRPRVTANKDARVFIIT-----YIVG-----YLVGVVFFH 326

AlAR_Homo_sapiens  SSMFRGSARITVSKQSSCTTARRSKSFLQVCCCVGPSFSLDKNHVFTIKVFTISLSENGEEV 466
AlAR_Bos_taurus   SSLFRGSARMAVARPSACTTARRSKSFLQVCCCLGPSFSGHGENHOFITIKVFTISLSENGEEV 466
CqAlAR_Crassostrea_gigas  VVYDVMAIRFDVSLTYSVVFVWTFYFNSTLNFFLYNFSSEFRTAFQVLRGRIR----- 382

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Table 1

Sequences of the primers used in the experiment.

Primer	Sequence (5'-3')	Sequence information
P1 (forward)	ATGAATGTATCGGCGTCCTCCG	CgA1AR-1 specific primer
P2 (reverse)	CTATCGATGTCTTCCTCTCAGTACATCCTG	CgA1AR-1 specific primer
P3 (forward)	TTACAAAAGCTAACAAAGACGCTC	Real-time CgA1AR-1 primer
P4 (reverse)	GTCAACCAAAATACTACTGAATAC	Real-time CgA1AR-1 primer
P5 (EF-RTF)	AGTCACCAAGGCTGCACAGAAAG	Real-time CgEF primer
P6 (EF-RTR)	TCCGACGTATTTCTTTGCGATGT	Real-time CgEF primers
P7 (forward)	CGCAATGGTCGCTTGGTGGTC	Real-time TNF (CGI_10005109) primer
P8 (reverse)	CGTAGGGGCGGAAGGTCTCG	Real-time TNF (CGI_10005109) primer
P9 (forward)	ATTGGAGCACCTGGAGGATAAG	Real-time TNF (CGI_10006440) primer
P10 (reverse)	CAGTCTTCCGTGCTGGTATTTTC	Real-time TNF (CGI_10006440) primer
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	pMD18-T simple vector primer
RV-M	GAGCGGATAACAATTTACACAGG	pMD18-T simple vector primer
P11 (forward)	GGCCACGCGTCGACTAGTACT ₁₇	Oligo(dT)-adaptor

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