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Bursicon homodimers induce innate immune by activating the expression of anti-microbial peptide genes in the shrimp *Neocaridina heteropoda*

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ABSTRACT

Bursicon is a neurohormone belonging to the cystine knot protein family. It consists of two subunits (burs α and burs β) and plays a pivotal role in cuticle tanning and wing expansion in insects. Recent studies show that homologous crustacean bursicon stimulates cuticle thickening and granulation of hemocytes in the crab *Callinectes sapidus*. Here we investigate whether bursicon homodimers function in immunoprotective defense systems of shrimp. We found that abdominal ganglion was the main neurohemal release site of bursicon in *Neocaridina heteropoda*. Bacterial infections induced overexpression of burs α (bursicon α) and burs β (bursicon β). RNAi of burs α, burs β or both inhibited the expression of anti-microbial peptide (AMP) genes. Treating shrimp adults with r-bursicon (recombinant bursicon) homodimers led to up-regulation of three AMP genes. Besides, through the induced AMPs, r-bursicon homodimers enhanced the bacteriostasis of shrimp *in vivo* and *in vitro*. These findings demonstrate a novel function of bursicon in crustacean that it induces innate immune via up-regulating the expression of genes encoding AMPs.

1. Introduction

The growth of arthropod involves a series of molts during which a new cuticle is formed to replace the old one (ecdysis) [1]. The newly formed soft cuticle must be tanned (melanize and sclerotize) quickly to defend attack, injury and infection. In insects the neurohormone bursicon mediates the tanning process of the newly eclosed adults. Bursicon was first found by Fraenkel, Hsiao and Cottrell in 1962. They ligated the cervix of the *Calliphora erythrocephala* which just finished the eclosion (a classical procedure that effectively separated brain endocrine factors from the rest of the body), causing the epidermis of their chest and abdomen not to be tanned. The hemolymph of the normally developing individuals was injected into the body of these abnormal flies, and the epidermis of the chest and abdomen continued to be tanned [2,3]. Therefore, Fraenkel and Hsiao believed that there was a substance that was involved in epidermal tanning in hemolymph and named it bursicon [4]. Bursicon was a heterodimer neuropeptide composed of two cystine knot proteins, burs α and burs β. Expression profile studies showed that burs α or burs β was expressed in a set of neurosecretory cells (NSCs) from several insects, including *Drosophila melanogaster*, *Manduca sexta*, and *Musca domestica* [5–7]. It elicited cuticle tanning through cAMP/PKA signaling pathway and a key enzyme tyrosine hydroxylase [5,8]. Aside from regulating cuticle tanning, bursicon also functioned in wing expansion [6,9,10], integumentary development [11], and migration of the border cells in *D. melanogaster* during oogenesis [12].

In addition to forming the heterodimer responsible for cuticle tanning [5,8], studies showed that bursicon subunits also form burs α-α and burs β-β homodimers *in vitro* [5,13]. There were some reports suggesting the additional roles of bursicon. Using gene chip analysis, 87 downstream genes were found to be regulated by bursicon in the neck-ligated fruit flies. Most of the known genes were related to the tanning of the epidermis and the extension of the wings. However, there were 7 genes involved in immune regulation, including three of the Turandot gene family, two from the anti-microbial peptide (AMP) family, and the remaining two from the immunoglobulin gene and the silkworm anti-microbial peptide family [13]. An and others injected recombinant BURS and PBURS (also termed bursicon α and β) homopolymers into the *D. melanogaster* which were pre-ligated in the neck. They found that some immune related genes increased, besides, the degree of immune response varies with the dose and time of injection of recombinant protein. The homodimers activated a transcriptional factor, Relish, through a DLGR2-independent mechanism. Moreover, the study showed that the homogenate of the flies injected with both homodimers could inhibit the growth of the Gram-negative bacteria *Escherichia coli*. It was further conjecture that the homodimers formed by the BURS

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subunit and PBURS subunit might be combined with a new receptor through the IMD pathway and participated in the regulation of the immune process [14].

Compared with the studies in insects, there were few reports about bursicon in crustaceans till now. Bursicon was identified in the water flea, *Daphnia arenata*, the green shore crab, *Carcinus maenas* and the European lobster, *Homarus gammarus*. The thoracic ganglia complex (TGC) especially the terminal abdominal ganglia (AG 6–8) was identified as the largest bursicon producing region [15,16]. Chung et al. presented the evidence that bursicon controlled the cuticle sclerotization and the processes associated with it in the blue crab *Callinectes sapidus* [17]. On the other hand, bursicon was expressed constantly in cells and hemolymph throughout the molt cycle and life stages, besides, the copy numbers of *burs β* transcripts were at least 4-fold higher than *burs α* [17], which was also discovered in moth [6]. Since bursicon is conserved among arthropods, the hypothesis that bursicon homodimers act in immune system in crustacean seems to be reasonable. Here, we tested this hypothesis in the *Neocaridina heteropoda*.

2. Materials and methods

2.1. Animals

Neocaridina heteropoda in our study was purchased from a local aquatic product market in the city of Ningbo, China. Shrimps were kept in aerated water at 25 °C and acclimated for 2 weeks before processing. A 12 h light: 12 h dark photoperiod was used and the animals were fed once daily with pellet. All animal studies were performed with the approval of Tianjin Normal University Animal Ethics Committee.

2.2. RNA extraction and cDNA synthesis

Total RNAs were extracted from tissue samples including eyestalk, brain, thoracic ganglion, and abdominal ganglion using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. The first-strand cDNA was created using TransScript® one-step gDNA Remover and cDNA Synthesis SuperMix reverse transcription Kit (TransGen). Total RNA extraction and quality test were performed using Nanodrop 2000 (Thermo Fisher Scientific).

2.3. Quantitative PCR

β-actin was selected as the reference gene. According to the shrimp transcriptome data, gene primers (Qburs-α F/R, Qburs-β F/R, ALF F/R, Cru F/R, Lys F/R and β-actin F/R in Table 1) were designed using the software Primer 5.0. Real-time PCR was performed using Applied Biosystems StepOne Plus Real-time PCR System (Applied Biosystems). The procedure was performed as follows: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to analyze the expression pattern. Data were analyzed by the one-way ANOVA. Gene expression level with a P value < 0.05 was assigned as significantly different.

2.4. Preparation and injection of double-stranded RNA

Partial fragments of Nhbursα and Nhbursβ were cloned (using primers dsbursα F/R and dsbursβ F/R in Table 1) and ligated into pET-32a-c that was genetically engineered with restriction enzyme sites of EcoR I and Xba I. Then the recombinant plasmids were transformed into HT115 and induced. After the double-stranded RNA of bursicon-α and bursicon-β was prepared by HT115, We injected into abdominal muscle using a microinjection system (Nanoliter 2010, World Precision Instruments), with the control group injected with dsGFP. The injected dose was 1μg/150 mg. Then gene interference efficiency was detected by Real-time PCR.

Table 1
Primers used in the study.

Primers	Length (bp)	Primer sequences (5′-3′)
Qburs-α F	20	ATTCCAAGCCCATTCCATCC
Qburs-α R	20	TCCCTCTTCAACATCGGTGC
Qburs-β F	23	TCCATCAACCATACACATTTCCA
Qburs-β R	20	ATCCAGTCAAGCGATTTCCG
β-actin F	21	TGTGACGATGAAGTAGCAGCA
β-actin R	22	AATCTTTCTGACCCATTCCAAC
ALF F	21	GTTGGTCTCTGGAATCTTGGC
ALF R	19	CGAAGTCTCGGGTCGTCAA
Cru F	22	TGGCATCATTCCAGGACTAACT
Cru R	19	AGGAGGACATTGAGGGCGT
Lys F	23	CAGAACAGAGACCTGGACGAATA
Lys R	23	TGATACATAATGGGAACACGGAC
dsbursα F	27	GCTCTAGAATTCCAAGCCCATTCCATC
dsbursα R	27	GGAATTCGTTCCCTCTTCAACATCGGT
dsbursβ F	28	GCTCTAGATCACGGTGGGTATGGATTGT
dsbursβ R	27	GGAATTCGTGTATTGACGGAGGGTTGG
bursicon-α F	26	ATGCACTTAAAGAAGGTTAATACAAG
bursicon-α R	22	TTAAATGAAGGGTACGTTTCCC
bursicon-β F	24	ATGTGGTCACGGTGGGTATGGATT
bursicon-β R	26	TTATCGTGTGGAATCACCACATTTGG

2.5. Recombinant plasmid construction of burs α and burs β

The PCR primers used for cloning the specific target DNA fragments of burs α and burs β were designed by the primer 5.0 (bursicon-α F/R and bursicon-β F/R in Table 1). The *BamH* I restriction site was added to the 5′ end of the upstream primers and the *Hind* III restriction site was added to the 3′ end of the downstream primer. The PCR program was performed as follow: 94 °C for 4 min, followed by 94 °C for 30 s, 61(burs-α)/64(burs -β) °C for 30 s, 72 °C for 30s for 35 cycles and 72 °C for 10 min. Prime STAR HS DNA Polymerase (Takara) was used to ensure high fidelity and accuracy during amplification. Followed by digestion with enzymes Hind III and Nde I (Takara), the amplified fragment of burs-α and burs-β was inserted into the pET-21a(+) vector. The recombinant plasmid was transformed into competent Rosetta (DE3) (Biovector 610,066) cells for recombinant protein expression. Positive clones were screened by PCR and the extracted plasmids were sequenced to confirm the result.

2.6. Expression and purification of the recombinant burs α and burs β

The constructed plasmids encoding recombinant His-tagged burs α and burs β (rNhbursα and rNhbursβ) were transformed into Rosetta cells. Positive transformants grown in LB medium (kanamycin and spectinomycin contained) at 37 °C were induced with 1 mM IPTG at OD_{600} = 0.6 for 6 h. Bacteria cells were harvested by centrifugation at 4000 rpm for 10 min at 4 °C. The fragmentation buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1 mg/ml lysozyme, pH 7.4) was added to the cells obtained, followed by the sonication. Then the samples were centrifuged at 10000 rpm for 10 min at 4 °C. The supernatants were retained (rNhbursβ existed) and the pellets were re-suspended in washing buffer (300 mM KCl, 50 mM KH_2PO_4, 5 mM imidazole and 1 M urea) and washed three times, followed by dissolving in binding buffer (300 mM KCl, 50 mM KH_2PO_4, 5 mM imidazole with 8 M urea).

rNhbursα and rNhbursβ were then purified using Ni-NTA His-bind column (GE). Then the glutathione redox system was used to dialysate the purified recombinant Bursα protein. The components of the reconstituted solution were: 50 mM Tris-Cl, 2 mM GSH, 0.2 mM GSSG, 0.1 M arginine. The urea concentrations were 8, 6, 4, 3, 2, and 1 M, respectively. Each concentration of urea was dialyzed for at least 4 h. Finally, the Bursα protein was dialyzed into a 1 × PBS solution. Bursβ was directly dialyzed into 1 × PBS. The two recombinant proteins were subjected to reductive and non-reducing SDS-PAGE electrophoresis (no

reducing agent was added to non-reducing gel). The monomers remaining were removed using the ultra-30kD centrifugal filters (Merckmillipore). The endotoxin in purified protein was tested quantitatively using chromogenic *Limulus* amebocyte lysate reagent (GeneScript). Endotoxin concentrations were 0.16 and 0.20 EU/ml, respectively. Preliminary experimental results showed that these trace endotoxin remaining would not affect the subsequent experiments (data not shown).

2.7. Bursicon homodimer treatment and bioassay

A total of 180 shrimps were selected, each of which was about 1.5–2 cm in size, divided into 3 groups. The r-bursicon homodimers were injected into the abdominal muscle (80 ng protein), using a microinjection system (Nanoliter 2010, World Precision Instruments). Control group were injected with PBS. At the indicated times post injection (0.5 h, 1 h, 3 h), abdominal nerves were obtained and stored in liquid nitrogen. Total RNA was extracted, qRT-PCR analysis was performed to detect differences in gene expression.

2.8. Bacterial inhibition assay

Shrimps injected with proteins were placed in a 200 μl centrifuge tube, with a hole in the bottom. Then this tube was put into the 1.5 ml centrifuge tube which was pre-loaded with the extraction buffer (0.05 mol/l ammonium acetate, pH 5.0, 35 μg/ml PMSF, 2% mercaptoethanol). The tube was centrifuged at 4000 rpm for 20 min at 4 °C. The body fluid acquired was boiled for 10 min to remove the internal bacteria, followed by the centrifugation at 12000 rpm for 20 min at 4 °C. Supernatants were mixed with the typical G^- and G^+ bacterial strain (*Escherichia coli, Bacillus thuringiensis*) at10^5 cfu/ml (*E. coli*) and 10^4 cfu/ml (*B. thuringiensis*), respectively. The strains used were all pathogenic ones isolated from the diseased shrimps and identified through 18s rDNA analysis. After incubation at 37 °C for 3 h, the mixtures were plated and the colonies were counted after overnight incubation.

2.9. Analysis of mortality rate

Shrimps were injected with PBS, rNhbursα (100 ng) and rNhbursβ (100 ng). 3 h later, 150 nl of 10^9 cfu/ml *E. coli* or 10^9 cfu/ml *B. thuringiensis* were injected for immune challenge. Then the death number was recorded in each group every day and the mortality statistics were done.

3. Results

3.1. Identification and distribution of bursicon in the neuroendocrine system

The cDNAs of Nhburs α (*Neocaridina heteropoda* burs α) and Nhburs β (*Neocaridina heteropoda* burs β) had been acquired in our previous study (GenBank accession number: MG766223 and MG766224, respectively). Since it had been studied in other arthropod that bursicon, as an endocrine hormone, mainly expressed in nervous system, we investigated their expression profiles in the *N. heteropoda*. Using cDNAs from abdominal ganglion (AG), thoracic ganglion (TG), brain (Br), and eyestalk (ES) as templates, and the *β-actin* gene as an internal reference gene, the expression of *Nhburs α* and *Nhburs β* were measured by the fluorescence quantitative PCR. The result showed that both *Nhburs α* and *Nhburs β* expressed mostly in the abdominal ganglion, secondly in the thoracic ganglion. The relative expression levels in the brain and eyestalk were extremely low (Fig. 1). Besides, *Nhburs β* had a much higher expression than α in AG but was surpassed by α in TG.

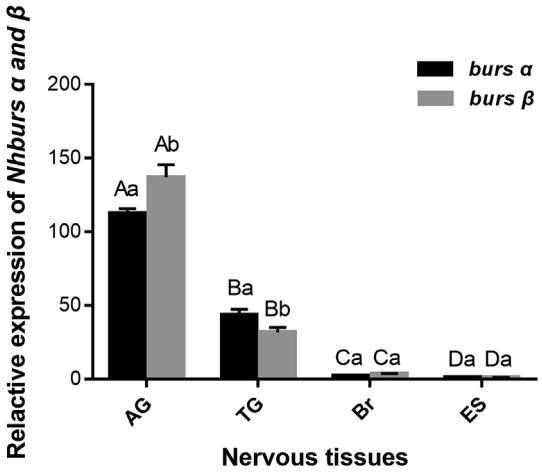


Fig. 1. Tissue expression of *burs α* and *burs β* determined by Q-PCR. *Nhburs* is expressed mainly in AGs and TGs but nearly undetectable in brains or ES. About three times more *Nhburs* is expressed in AGs compared with TGs. β-actin is used as an internal control. Data denoted with different lowercase letters indicate significant differences within groups; the capital letters indicate significant differences between groups ($P < 0.05$, one-way ANOVA followed by Duncan's analysis); mean ± s.d. (n = 6). AG, abdominal ganglion; TG, thoracic ganglion; Br, brain; ES, eyestalk.

3.2. Bacterial infections induced overexpression of Nhburs α and Nhburs β

Expressive differences in the same neuroendocrine organs indicated that *Nhburs α* and *β* possibly participated in other physiological processes, in the form of homodimers. We detected whether they involved in the immune responses. The shrimps were injected with $Gram^-$ bacteria *E. coli*, $Gram^+$ bacteria *B. thuringiensis* and PBS, as a negative control. Half day and one day after the injection, the mRNA expression levels of *Nhburs α and β* were analyzed by quantitative PCR. Compared with the control group, expression of *Nhburs α and β* mRNA in shrimps injected with *B. thuringiensis* increased at 0.5 and 1 day. However, there was no significant change in the expression level of *Nhburs α* under the stimulation of *E. coli*, regardless of stimulation time (Fig. 2).

3.3. Depletion of bursicon led to the expression of anti-microbial peptide genes decreased

To explore the function of bursicon in the immune system of *N. heteropoda*, dsRNA designed based on the cDNA sequences of *Nhburs α and β* were injected into the body of shrimps. RNAi efficiency was evaluated on the second and sixth day after injection. The result showed the expression of endogenous *bursicon α and β* were significantly knocked down compared with the control group (Fig. 3A and B). Besides, this interference effect lasted for at least 6 days, providing sufficient time for the following experiments.

We next investigated whether there was a correlation between the transcript levels of bursicon and representative anti-microbial peptide genes [Anti-lipopolysaccharide factor (ALF), Crustins, Lysozyme]. The cDNAs of three AMPs had been acquired in our previous study (GenBank accession number: ALF (MK044339), Crustin (MK044341) and Lysozyme (MK044340), respectively). As shown in Fig. 3C, compared with the control group, the reduction in *Nhburs α* transcript level induced significantly decreased expressions of all these three genes. Interestingly, this degree of decline was far less than that caused by RNAi of *Nhburs β* alone and *Nhburs α/β* in common. In particular, the expression of Crustins and Lysozyme were declined to the same level in shrimps injected with dsRNA of *Nhburs β* alone or both, demonstrating a more important role of *Nhbursβ*, in comparison to α, in the regulation of these two anti-microbial peptide genes.

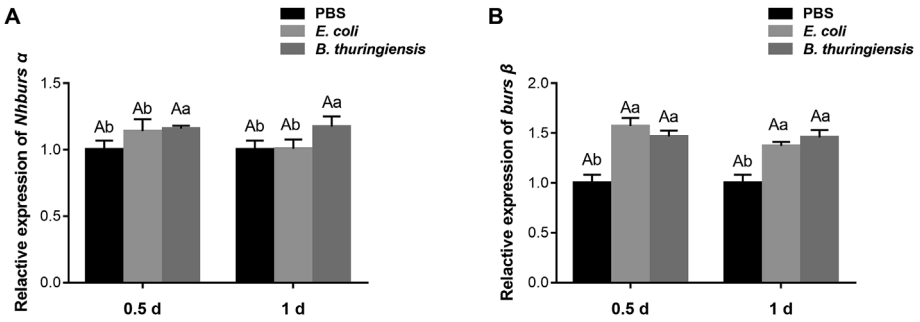


Fig. 2. Bacterial infections induced overexpression of Nhburs α and Nhburs β. Shrimp adults were injected with *E. coli*, *B. thuringiensis* and PBS, respectively. Half day and one day later, Nhburs α (A) and Nhburs β (B) gene expression was analyzed by qPCR.

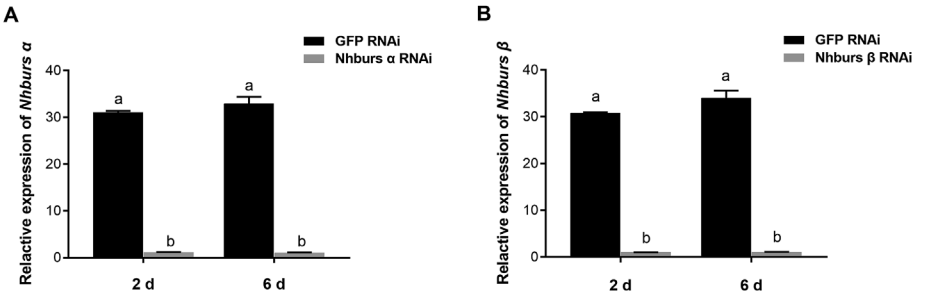
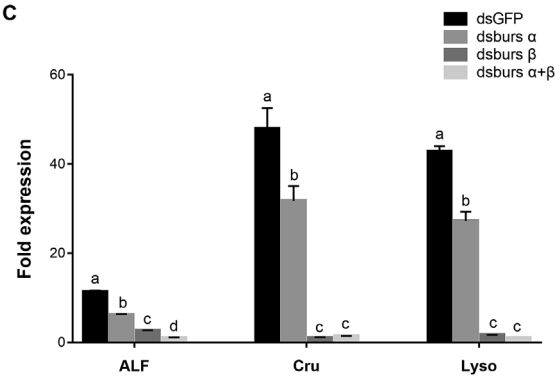


Fig. 3. Gene knockdown of bursicon by RNAi. Relative expression levels of *Nhburs α* (A) and *Nhburs β* (B) at different time after the dsRNA injection. (C) Changes in the expression level of three AMP genes in control, dsburs α, dsburs β and dsburs α/β groups. Values are presented as means ± s.d. (n = 10). Data labeled with different letters indicate a significant difference ($P < 0.05$) among treatments.



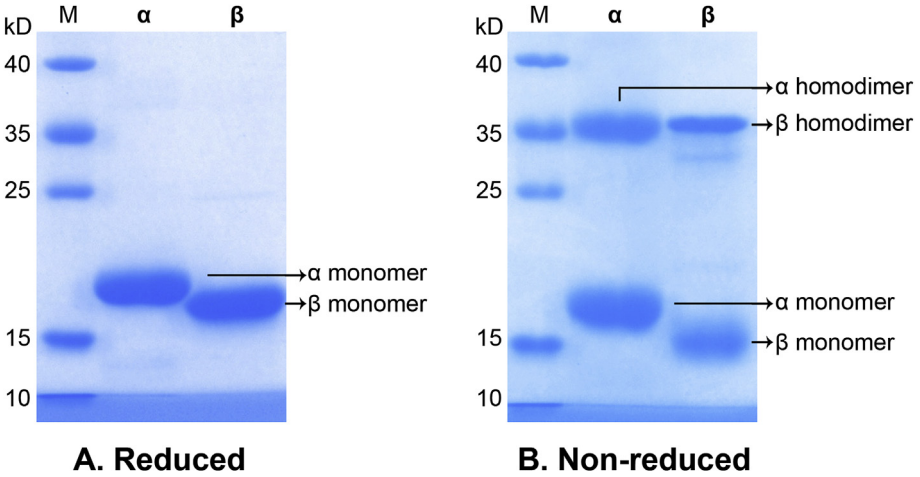


Fig. 4. r-bursicon proteins in reduced (A) and non-reduced (B) SDS-PAGE are identified. The positions of monomers, r-burs α-α and r-burs β-β homodimers are indicated. Numbers on the left indicate positions of molecular weight standards.

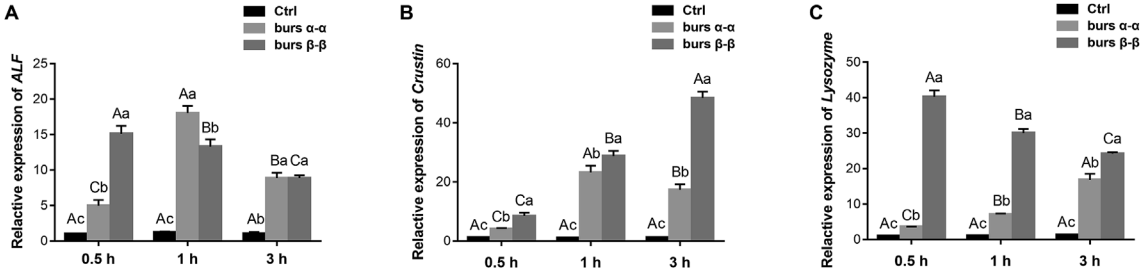


Fig. 5. Burs α-α and burs β-β treatments induce expression of AMP genes. Separate groups of shrimp adults were injected with 80 ng of r-burs α-α, r-burs β-β or PBS, respectively. At different time after the treatment, expression of the ALF (A), Crustin (B), and Lysozyme (C) genes were determined by qPCR.

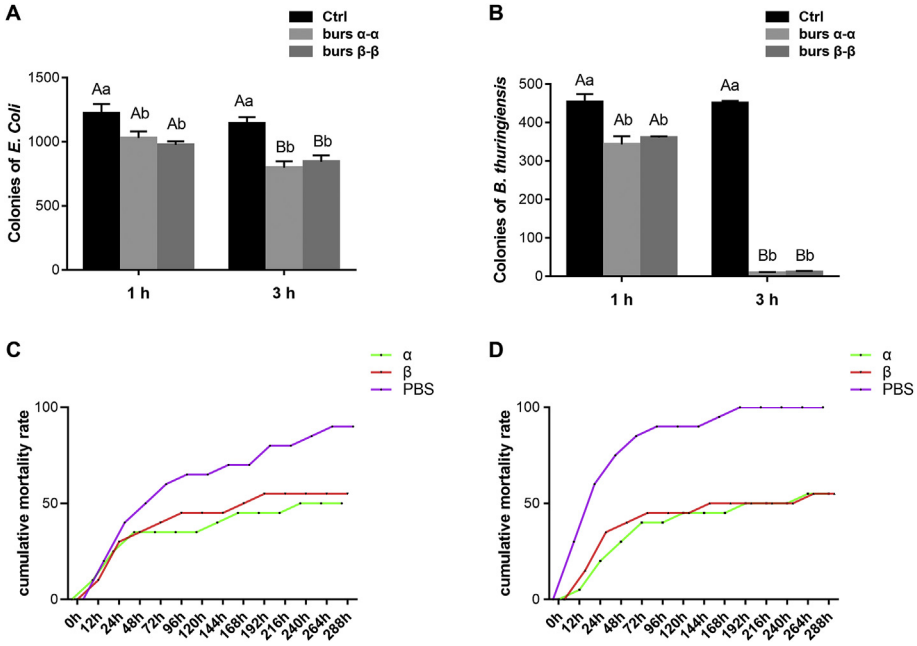


Fig. 6. Burs α-α and burs β-β treatments suppress bacterial growth and enhance the bacteriostasis in vitro and vivo. Shrimp adults were injected with r-burs α-α, r-burs β-β homodimers, or PBS, respectively. (A, B) The body fluid was acquired *in vitro* and challenged with indicated titers of *E. coli* and *B. thuringiensis* for 3 h before plating for colony count. The histograms show the means ± s.d, n = 6 biologically independent experiments. (C, D) 3 h following the r-protein injection, *E. coli* and *B. thuringiensis* were injected for immune challenge, respectively. Mortality was compared in both cases (n = 40).

3.4. Bursicon homodimers induced overexpression of anti-microbial peptide genes

The r-bursicon subunits were then expressed in prokaryotic system. When expressed individually, they form burs α-α and burs β-β homodimers. This was recognized by their molecular weights, because the molecular size of homodimers doubled in the non-reduced gel, compared to the reduced gel (Fig. 4A and B). This result was consistent with what had been reported by Luo et al. [18]. Some monomers did not form homodimers and were still present under non-reducing conditions (Fig. 4B).

To further verify that bursicon mediated the expression of AMP genes, we injected the r-burs α-α and β-β homodimers to the body cavity of shrimps. The results showed that relative to the control group, the expression levels of three AMP genes were substantially up-regulated 0.5 h–3 h post injection (Fig. 5). In detail, the expression of *ALF* gene and *Crustin* peaked at 1 h post injection of r-burs α-α and then decreased at 3 h, whilst the expression of *ALF* gene and *Lysozyme* gene increased at the beginning and then reduced till a certain value in the shrimps injected with r-burs β-β. The expression of *Crustin* presented a tendency to increase gradually with time post injection of r-burs β-β. The expression of *Crustin* and *Lysozyme* presented a tendency to increase gradually with time post injection of r-burs β-β and r-burs α-α, respectively. These data indicated that burs α-α and burs β-β homodimers mediated expression of AMP genes. We note that these two homodimers functioned asynchronously.

3.5. Bursicon homodimers enhanced the bacteriostasis of shrimp

We next considered whether the AMP genes up-regulated by r-bursicon had a good promotion effect on resisting bacteria. Shrimps injected with r-burs α-α, β-β homodimers or blank vector transfected sample (control) were collected and homogenized at 1 h and 3 h. The supernatants were incubated with bacteria for 3 h. Bacterial inhibition assays showed that the growth of *E. coli* and *B. thuringiensis* was significantly inhibited in samples treated with these two kinds of homodimers. Besides, r-burs β-β injected group showed stronger antibacterial effect, with 40% *E. coli* killed (Fig. 6A) and almost no *B. thuringiensis* survived (Fig. 6B), compared to the control group. This result demonstrated that burs α-α and β-β homodimers effectively prevented bacterial infection and promoted immune defense via up-regulated AMP genes.

Following *E. coli* challenge infection by injection, shrimps died rapidly in 24 h after injection (Fig. 6C). However, shrimps pre-injected with r-burs α-α and β-β homodimers showed much less mortality rate after 24 h post-challenge and stabilized at 50% 8 days after recording, compared to the control group which showed about 90% mortality finally. Infection of shrimp with *B. thuringiensis* showed the similar trend (Fig. 6D). Mortalities of protein injected shrimps commenced from the beginning but stopped at day 8, after reaching 50%, by then all shrimps in the control group were dead.

4. Discussion

Bursicon belongs to the cystine knot protein family, including vertebrate glycoprotein hormones, growth factors et al. [19]. All these members consist of two subunits which form heterodimes executing physiology functions. Some also form homodimers and function differently. It was reported that each of bursicon subunit could form a homodimer in several insect species [5]. However, in crustacean, whether bursicon has the form of homodimer and the same function as reported are still unknown. Here, the data in this paper showed that injection of r-bursicon α-α and β-β homodimers upregulated AMP genes, demonstrating that bursicon homodimers functioned in mediating AMP gene transcription *in vivo*. This result was verified by means of RNAi. Decreased burs α, burs β or both led to low levels of AMPs. Besides, exogenous protein injection enhanced the bacteriostasis of shrimp. Taken together, these points suggested that bursicon homodimers acted in innate immune of crustacean, by mediating expression of AMPs, as reported in *D. melanogaster* [14] and *A. aegypt*i [20].

We also noted that *Nhbursα* was not significantly responsive to *E. coli* infection, besides, the decrease of AMP gene expression caused by interfering of burs α alone was not as good as interfering of β or both, This might indicate that burs β played a greater role in the up-regulated control of AMPs than burs α. In addition, injection of recombinant proteins also showed that burs β-β homodimer caused a greater increase of AMP gene expression than burs α-α homodimer. Nervous tissue expression characteristics showed that in abdominal ganglion, which appeared to be the main biosynthesis sites of bursicon, *burs β* transcripts outnumbered *burs α* ones. Interestingly, *burs β* transcripts also outnumbered *burs α* transcripts in the blue crab, *C. sapidus* [17]. We infer that in the case of unequal-probability expression of bursicon subunit genes, large abundance of *burs β* transcripts form burs β-β homodimers and play more important roles than burs α-α homodimers, in the process of inducing innate immune.

The findings in this paper show that a novel important hormonal action of bursicon in crustacean is mediating innate immune via upregulating AMPs. This extends the physiological functions of bursicon beyond its classical roles in cuticle tanning and wing expansion in insects. The multiple functions reported to date may explain the high conservation of bursicon in arthropods and provide us angles in deeply discovering its mechanism.

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References

[1] L.M. Riddiford, P. Cherbas, J.W. Truman, Ecdysone receptors and their biological actions, Vitam. Horm. 60 (1) (2000) 1–73.
[2] C.B. Cottrell, The imaginal ecdysis of blowflies detection of the blood-bone darkening factor and determination of some of its properties, Bioorg. Med. Chem. 39 (1) (1962) 67–73.
[3] G. Fraenkel, C. Hsiao, Hormonal and nervous control of tanning in the fly, Science 138 (3536) (1962) 27–29.
[4] G. Fraenkel, C. Hsiao, Bursicon, a hormone which mediates tanning of the cuticle in the adult fly and other insects, J. Insect Physiol. 11 (5) (1965) 513–556.
[5] C.W. Luo, E.M. Dewey, S. Sudo, J. Ewer, S.Y. Hsu, H.W. Honegger, A.J. Hsueh, Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2, Proc. Natl. Acad. Sci. U.S.A. 102 (8) (2005) 2820–2825.
[6] L. Dai, E.M. Dewey, D. Zitnan, C.W. Luo, H.W. Honegger, M.E. Adams, Identification, developmental expression, and functions of bursicon in the tobacco hawkmoth, *Manduca sexta*, J. Comp. Neurol. 506 (5) (2010) 759–774.
[7] S. Wang, S. An, Q. Song, Transcriptional expression of bursicon and novel bursicon-regulated genes in the house fly *Musca domestica*, Arch. Insect Biochem. Physiol. 68 (2) (2010) 100–112.
[8] F.M. Mendive, L.T. Van, S. Claeysen, J. Poels, M. Williamson, F. Hauser, C.J. Grimmelikhuijzen, G. Vassart, J. Vanden Broeck, Drosophila, molting neurohormone bursicon is a heterodimer and the natural agonist of the orphan receptor DLGR2, FEBS (Fed. Eur. Biochem. Soc.) Lett. 579 (10) (2005) 2171–2176.
[9] J.E. Natzle Jr., KJ, M.M. Green, Bursicon signaling mutations separate the epithelial-mesenchymal transition from programmed cell death during Drosophila melanogaster wing maturation, Genetics 180 (2) (2008) 885–893.
[10] J. Huang, Y. Zhang, M. Li, S. Wang, W. Liu, P. Couble, G. Zhao, Y. Huang, RNA interference-mediated silencing of the bursicon gene induces defects in wing expansion of silkworm, FEBS (Fed. Eur. Biochem. Soc.) Lett. 581 (4) (2007) 697–701.
[11] H. Bai, S.R. Palli, Functional characterization of bursicon receptor and genome-wide analysis for identification of genes affected by bursicon receptor RNAi, Dev. Biol. 344 (1) (2010) 248–258.
[12] L. Anllo, T. Schüpbach, Signaling through the G-protein-coupled receptor Rickets is important for polarity, detachment, and migration of the border cells in Drosophila, Dev. Biol. 414 (2) (2016) 193–206.
[13] S. An, S. Wang, L.I. Gilbert, B. Beerntsen, M. Ellersieck, Q. Song, Global identification of bursicon-regulated genes in Drosophila melanogaster, BMC Genomics 9 (1) (2008) 424-424.
[14] S. An, S. Dong, Q. Wang, S. Li, L.I. Gilbert, D. Stanley, Q. Song, Insect neuropeptide bursicon homodimers induce innate immune and stress genes during molting by activating the NF-κB transcription factor Relish, PLoS One 7 (3) (2012) e34510.
[15] D. Wilcockson, S. Webster, Identification and developmental expression of mRNAs encoding putative insect cuticle hardening hormone, bursicon in the green shore crab *Carcinus maenas*, Gen. Comp. Endocrinol. 156 (1) (2008) 113–125.
[16] J. Sharp, D. Wilcockson, S. Webster, Identification and expression of mRNAs encoding bursicon in the central nervous system of decapod crustaceans, Comp. Biochem. Physiol. A Mol. Integr. Physiol, 150 (3) (2008) 116–125.
[17] J.S. Chung, H. Katayama, H. Dircksen, New Functions of arthropod bursicon: inducing deposition and thickening of new cuticle and hemocyte granulation in the blue crab, *Callinectes sapidus*, PLoS One 7 (9) (2012) 1602–1603.
[18] C.W. Luo, E.M. Dewey, S. Sudo, J. Ewer, S.Y. Hsu, H.W. Honegger, A.J. Hsueh, Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2, Proc. Natl. Acad. Sci. U.S.A. 102 (8) (2005) 2820–2825.
[19] H.W. Honegger, E.M. Dewey, J. Ewer, Bursicon, the tanning hormone of insects: recent advances following the discovery of its molecular identity, J. Comp. Physiol. A Sens. Neural Behav. Physiol. 194 (12) (2008) 989–1005.
[20] H. Zhang, S. Dong, X. Chen, D. Stanley, B. Beerntsen, Q. Feng, Q. Song, Relish 2 mediates bursicon homodimer-induced prophylactic immunity in the mosquito *Aedes aegypti*, Sci. Rep. 7 (2017) 43163.