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Involvement of dopamine beta-hydroxylase in the neuroendocrine-immune regulatory network of white shrimp, *Litopenaeus vannamei*

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ABSTRACT

In shrimp, the biosynthesis of catecholamines, including dopamine and norepinephrine, is required for physiological and immunological responses against stress. Dopamine beta-hydroxylase (DBH), a copper-containing monooxygenase enzyme that plays an important role in catecholamine synthesis of the neuroendocrine regulatory network, was identified in *Litopenaeus vannamei*. In the present study, the potential role of DBH in the immunocompetence of *L. vannamei* was further estimated by depleting DBH by pharmaceutical inhibition of disulfiram and a gene silencing technique of *L. vannamei* DBH-double-stranded (ds)RNA (LvDBH-dsRNA). Immunocompetence was evaluated following the determination of the total hemocyte count, differential hemocyte count, phenoloxidase activity, respiratory bursts, superoxide dismutase activity, phagocytic activity, and the clearance efficiency as well as the susceptibility against *Vibrio alginolyticus* infection. At 30–120 min after shrimp had received disulfiram, they exhibited significantly reduced total hemocyte count, phenoloxidase activity of hemocytes in hemolymph, respiratory bursts of hemocytes in hemolymph and per hemocyte, phagocytic activity, clearance efficiency, and survival ratio against *V. alginolyticus* infection, compared to those injected with saline. In addition, the significantly lower total hemocyte count, phagocytic activity, clearance efficiency, and resistance to *V. alginolyticus* infection were observed in shrimp that received LvDBH-dsRNA at 3 days post injection compared to those injected with diethyl pyrocarbonate-water or non-targeting gene-dsRNA. The DBH depleted *L. vannamei* revealed immunosuppression and decreased the survival ratio to *V. alginolyticus* infection, which indicated that DBH played a crucial role in the neuroendocrine-immune regulatory network.

1. Introduction

In stress responses of crustaceans, the release of catecholamines, a class of biogenic amines, is the primary step followed by secondary effects including induction of hyperglycemia and suppression of immunity [1–3]. Such catecholamines as serotonin, dopamine, octopamine, and norepinephrine have been identified in decapods [4–7]. The release of catecholamines by decapods against environmental stress was reported, such as norepinephrine [1,8] and dopamine [8] in *Litopenaeus vannamei* or norepinephrine [9] and dopamine [3] in *Macrobrachium rosenbergii* exposed to thermal stress. Increased catecholamine levels were confirmed to cause immune suppression, with an increased susceptibility to pathogen infection [9–12], and a marked transient period of modulation in energy metabolism, osmoregulation, and respiratory responses when adapting to an environmental stressor [13,14].

In vertebrates, catecholamine neurotransmitters are synthesized in catecholaminergic neurons from tyrosine, via dopa, dopamine, and norepinephrine, to adrenaline (epinephrine), and there are four enzymes involved in the biosynthesis of adrenaline: (1) tyrosine 3-mono-oxygenase (tyrosine hydroxylase, TH); (2) aromatic L-amino acid decarboxylase (or DOPA decarboxylase); (3) dopamine β-mono-oxygenase (dopamine β-hydroxylase, DBH); and (4) noradrenaline N-methyltransferase (phenylethanolamine N-methyltransferase). Catecholamine neurons are involved in a wide range of brain functions, such as motor, neuroendocrine, biorhythm, feeding, mating, emotion, learning, and memory functions in the central nervous system and in sympathetic functions in

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peripheral noradrenaline neurons of mammals including humans [15]. In invertebrates, Zhou et al. [16,17] cloned and characterized DBH and aromatic L-amino acid decarboxylase in the mollusk scallop, *Chlamys farreri*, and their expressions in response to a bacterial challenge during ontogenesis were further reported [18]. In addition, activities of aromatic L-amino acid decarboxylase, DBH, and noradrenaline N-methyltransferase were used to evaluate the effects of a protein kinase C isoform on the pathway of biogenic amine biosynthesis in the marine mussel, *Mytilus galloprovincialis* [19].

The immune response in shrimp is an innate system including cellular and humoral defenses. Amparyup et al. [20] reviewed how the prophenoloxidase-activating system serves an important role as a non-self-recognition system that participates in innate immune responses through accompanying cellular responses via hemocyte attraction and inducing phagocytosis, melanization, cytotoxic reactant production, particle encapsulation, and the formation of nodules and capsules. To assess shrimp's immune response, Rodriguez and Le Moullac [21] indicated that the parameters of phenoloxidase activity, respiratory bursts (the generation of reactive oxygen intermediates), phagocytic activity, and the clearance efficiency are considered health marker. Mature hemocytes acting as a so-called "mobile immune-brain" was described in an invertebrate by Ottaviani et al. [22] and Ottaviani and Franceschi [23], and its cells were structured along an ancestral hypothalamic-pituitary-adrenal axis involving molecules similar to those detected in vertebrates and verified to have responses similar to those performed by macrophages in vertebrates [23,24]. In *L. vannamei*, TH [25,26] and DBH [9] were identified and characterized in hemocytes, and their roles in catecholamine biosynthesis after exposure to hypothermal stress was also confirmed. These results support hemocytes in shrimp possessing the function of a "mobile immune-brain".

Cheng et al. [8] reported that the DBH level of *L. vannamei* exposed to hypothermal stress significantly increased, and was accompanied by an increase in the NE level. In addition, DBH of hemocytes inhibited by disulfiram or silenced by LvDBH double-stranded (ds)RNA impaired the synthesis of NE from dopamine in hemolymph, suggesting that DBH of hemocytes in *L. vannamei* is involved in catecholamine biosynthesis. Chang et al. [9] indicated that hypothermal stress that induced acute modulation of immunity was associated with the release of hemolymph NE. We therefore assumed that DBH might possess the function of regulating the immunocompetence of *L. vannamei* hemocytes as a "mobile immune-brain". The aim of the present study was to explore DBH's function in immunocompetence through gene depletion and via pharmaceutical inhibition.

2. Materials and methods

2.1. Animals

Litopenaeus vannamei was supplied by a commercial farm in Pingtung, Taiwan, and shrimp were acclimated to laboratory conditions for 2 weeks in indoor fiberglass-reinforced plastic (FRP) tanks with aerated and flow-through seawater at the National Pingtung University of Science and Technology's Aquatic Animal Physiology and Immunology Laboratory. Shrimp were fed twice a day (08:00 and 17:00) with a formulated shrimp diet (Grobest Feed Company, Pingtung, Taiwan) at a rate of 5% of their body weight. During the acclimation and experimental periods, a water temperature of 28 ± 1 °C, pH of 7.5–7.8, dissolved oxygen level of 5.0–6.1 mg l^{-1}, salinity of 20‰, and photoperiod of 12 h L:D were maintained. Only shrimp in the intermolt stage were used for the study. The molt stage was determined by examining the uropoda in which partial retraction of the epidermis could be distinguished [27].

2.2. Experimental design

Inhibition of the DBH level and a decrease in LvDBH gene expression were detected in *L. vannamei* that received disulfiram for 30 and 120 min and LvDBH-dsRNA for 3 days [8], and therefore, to evaluate the potential role of LvDBH in regulating the immunity of *L. vannamei*, disulfiram for pharmaceutical inhibition and LvDBH-dsRNA for gene silencing tests were used as described by Cheng et al. [8]. In the present study, the susceptibility, phagocytic activity and clearance efficiency, and immunocompetence assessment in gene silencing test was assessed with 120, 30, and 30 shrimp, and those in pharmaceutical inhibition tests were 90, 30, and 30 shrimp, respectively. The shrimp sampled for the determinations are described below, and in total, 330 shrimps were used in this part of the study.

2.3. Susceptibility test against Vibrio alginolyticus infection

The bacterium *V. alginolyticus* isolated from diseased *L. vannamei* collected from farms in Pingtung, Taiwan, which displayed symptoms of anorexia, inactivity, poor growth and necrotic musculature, was used in this study [28]. To determine the susceptibility of *L. vannamei* injected with disulfiram or LvDBH-dsRNA to *V. alginolyticus* infection, all test and control groups were comprised of 10 shrimp each in triplicate. *Vibrio alginolyticus* was prepared as described by Mapanao and Cheng [25], and the concentration of the bacterial stock solution was 5×10^7 colony-forming units (cfu) ml^{-1} for the susceptibility test and 3×10^8 cfu ml^{-1} for the phagocytic activity and clearance efficiency assays. At 3 days post injection of LvDBH-dsRNAs, non-targeting gene-dsRNA and diethyl pyrocarbonate-water (DEPC-H_2O) or 120 min after the injection of disulfiram, 20 μl of a *V. alginolyticus* bacterial suspension (5×10^7 cfu ml^{-1}) was injected into the ventral sinus of the cephalothorax resulting in 10^6 cfu $shrimp^{-1}$. Shrimp that received LvDBH-dsRNA for 3 days or disulfiram for 120 min and then were injected with saline served as the blank control. The mortality was recorded for 7 days.

2.4. Effects of disulfiram on the immunocompetence of L. vannamei

Litopenaeus vannamei shrimp (16–17 g) obtained and acclimated as described above were individually injected in the ventral sinus of the cephalothorax with a 0.2 mg ml^{-1} disulfiram (Sigma-Aldrich, St Louis, MO, USA) solution (20 μl) to reach a final dose of 4 μg $shrimp^{-1}$ [9]. Shrimp that received saline (20 μl) served as the control group. There were two treatments (saline and disulfiram) with two sampling times (30 and 120 min). Six shrimp for each treatment and time were used for these studies. In addition, another six shrimp without treatment were used as the initial group (0 h). Immune parameters of the total hemocyte count (THC), granular cells, semigranular cells, hyaline cells, phenoloxidase activity of hemocytes in hemolymph, phenoloxidase activity per granulocyte (the sum of semigranular cells and granular cells), respiratory bursts of hemocytes in hemolymph, respiratory bursts per hemocyte, and superoxide dismutase activity were determined. Following the same experimental design, another 30 shrimp were used for the phagocytic activity and clearance efficiency assays against *V. alginolyticus*.