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Induction of ecdysteroidogenesis, methyl farnesoate synthesis and expression of ecdysteroid receptor and retinoid X receptor in the hepatopancreas and ovary of the giant mud crab, *Scylla serrata* by melatonin

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ABSTRACT

Melatonin, a chronobiotic molecule, is known to modulate several physiological functions in crustaceans including reproduction, molting and glucose homeostasis. In our earlier studies (Sainath and Reddy, 2010a), we observed hyperglycemia in crabs after melatonin administration and concluded that melatonin is another crustacean hyperglycemic hormone. In the current study, we have further examined the role of melatonin in regulating the levels of methyl farnesoate and ecdysteroid in the giant mud crab *Scylla serrata* and determined that melatonin indeed is a reproductive hormone. Further, we have determined partial nucleotide sequences of retinoid X receptor (*RXR*) and ecdysone receptor (*EcR*) in *S. serrata* and also studied the effect of melatonin on expression of these genes. Cloned *RXR* and *EcR* possess high sequence similarity with other Brachyuran genes. Administration of melatonin elevated circulatory methyl farnesoate (MF) and ecdysteroid levels in crabs. Since MF and ecdysteroid act through RXR and EcR respectively and these receptors are involved in the regulation of reproduction in crustaceans, we measured the expression levels of *RXR* and *EcR* in hepatopancreas and ovary after melatonin administration. The expression levels of both *RXR* and *EcR* increased significantly in the hepatopancreas and ovary of melatonin injected crabs when compared to the controls. *In vitro* culture of mandibular organ (MO) and Y-organ (YO) in the presence of melatonin resulted in a significant increase in the secretion of methyl farnesoate and ecdysteroid respectively. From the above studies it is clear that melatonin stimulates YO and MO, resulting in increased synthesis of ecdysteroids and methyl farnesoate, and thereby inducing reproduction in *S. serrata*.

1. Introduction

Melatonin is a well known vertebrate pineal hormone involved in circadian and seasonal rhythms. Melatonin is synthesized in the dark because the enzymes involved in melatonin biosynthesis are scotoactive. Hence melatonin is called a chronobiotic molecule or hormone of darkness. Apart from pineal gland, it is also identified in different tissues of vertebrates such as the retina, Harderian gland and gastro-intestinal tract (Tan et al., 2014). The hormone melatonin is well-studied in vertebrates and has activity as an autocoid, a hypnotic, and a biological modifier, and is involved in general homeostasis, neurohumoral balance, thermoregulation, sleep–wake patterns, and reproduction through synergic actions with other hormones and neuropeptides (Cassone et al., 1993; Golombek et al., 1996; Hyde and Underwood, 1995; Reiter, 1991; Rowe and Kennaway, 1996; Stankov et al., 1991; Tan et al., 2003). In vertebrates, melatonin acts through melatonin receptors; of these, MT1, and MT2 are G-protein coupled receptors and MT3 is quinine reductase (Nosjean et al., 2000). Further, it was demonstrated that melatonin also binds to retinoid receptors and cytoplasmic proteins such as calmodulin and tubulin (Wiesenberg et al., 1998).

In invertebrates, the role of melatonin has not been studied extensively, though it has been detected in nearly every organism tested, including crustaceans (Balzer et al., 1997; Maciel et al.,

Abbreviations: RXR, retinoid X receptor; EcR, ecdysone receptor; MF, methyl farnesoate; MO, mandibular organ; YO, Y-organ; BLAST, basic local alignment search tool.

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2008; Tilden et al., 1997, 2001; Withyachumnarnkul et al., 1992, 1999). A recent study by Cary et al. (2012) showed that melatonin possesses both neuritogenesis and neuroprotective effects in crustacean X-organ cells. Besides direct hyperglycemic action (Sainath and Reddy, 2010a), melatonin has also been shown to influence X-organ mediated functions like locomotion (Tilden et al., 2003a, 2003b) and limb regeneration (Tilden et al., 1997). We have reported earlier that administration of melatonin induced precocious vitellogenesis in the fresh water edible crab *Oziothelphusa senex senex* (Sainath and Reddy, 2010b).

In crustaceans, methyl farnesoate and ecdysteroid are well known hormones that regulate reproduction. Methyl farnesoate and ecdysteroids are produced by the mandibular organ (MO) and Y-organ (YO) respectively. A study by Hopkins et al. (2008) authenticated that MF and ecdysteroids acts through the retinoid X receptor (RXR) and ecdysteroid receptor (EcR) respectively. Nagaraju et al. (2011) reported that silencing of *RXR* resulted in decreased vitellogenesis in *Carcinus maenas*. Martin et al. (2001) showed that ecdysteroid signaling plays a key role in induction of vitellogenesis in the mosquito *Aedes aegypti*. The role of these receptors in the regulation of reproduction is well established in crustaceans (Nagaraju et al., 2011).

Since melatonin, identified in crustaceans, is known to bind to the RXR, and RXR is involved in vitellogenesis, the present study was designed to establish the role of melatonin in the regulation of circulating methyl farnesoate and ecdysteroids, and in the expression of RXR and EcR in the hepatopancreas and ovary of the giant mud crab *Scylla serrata*.

2. Material and methods

2.1. Collection and maintenance of animals

Indian giant mud crabs, *S. serrata* (15 ± 2 cm in carapace width; 110 ± 5 g wet weight), were collected from the Nellore coast, Andhra Pradesh, India. The crabs were kept in large aquaria with continuous aeration and acclimatized to laboratory conditions for one week under constant salinity (25 ± 1 ppt), pH (7.2 ± 0.1), and temperature (23 ± 2 °C). During the experimentation, the crabs were fed daily with fish flesh. Feeding was stopped 24 h before experimentation.

2.2. Experimental design

Intermolt (stage C_4) female crabs were selected and divided into three groups of ten crabs each. Melatonin (Sigma Chemical Co., St. Louis) was first dissolved in absolute ethanol then subsequently diluted in crustacean saline (Pantin, 1934) to bring the ethanol concentration to 1%. The animals in the first group served as controls and were used for analysis at the beginning of the experiment. The crabs in the second group served as concurrent controls and were treated the same as the experimental groups, but received 20 μL of crustacean saline (containing 1% ethanol). The crabs in the third group were injected with melatonin (0.4 ng/gram body weight) on the 1st, 7th, 14th and 21st day of experiment. Crabs in groups 2 and 3 were euthanized and sampled on 28th day to evaluate the effect of melatonin. Experimental duration and melatonin dose regimen were selected based on our previous studies (Sainath and Reddy, 2011).

2.3. In vitro culture of MOs and YOs

MOs and YOs were isolated from ice-anesthetized intermolt (stage C_4) female crabs in pre-vitellogenic stage and washed in ice-cold sterile crab saline (containing 30.68 g sodium chloride, 0.989 g potassium chloride, 1.375 g calcium chloride, 2.359 g magnesium chloride and 0.222 g sodium bicarbonate in 1 L of double distilled water; pH 7.0) for 30 min. Since YOs and MOs did not survive for more than 6 h in crab saline, modified medium 199 was used to culture the YOs and MOs. Modified medium 199 contains 9.6 g of M199 powder (with L-glutamine and Earle's salts), 2.2 g of $NaHCO_3$ in 1000 mL crab physiological saline (containing 205.33 mmol/L NaCl, 5.36 mmol/L KCl, 2.46 mmol/L $MgCl_2{\cdot}6H_2O$, 15.3 mmol/L $CaCl_2{\cdot}2H_2O$, 5 mmol/L maleic acid, 5 mmol/L Tris; pH 7.4). Though medium 199 was devised for vertebrate tissues, YOs and MOs cultured in modified medium 199 did not show any symptoms of cell toxicity/injury. The incubated glands were observed under a microscope and no significant changes were detected in the size and shape of the cells. The cells were normal even after 72 h incubation in the culture medium. Incubation of MOs/YOs in culture medium resulted in MF/ecdysteroid secretion in a time-dependent manner up to 72 h, whereas incubation of glands up to 96 h resulted in no further increase in secretion (Nagaraju et al., 2006). Hence, we restricted our *in vitro* studies to 72 h. Individual MOs and YOs were subsequently cultured in 24-well tissue culture plates in 2 mL of the modified medium 199, containing fetal bovine serum (10%), penicillin-G (12.5 μg/mL), streptomycin (12.5 μg/mL) and either 50 μL of melatonin (3.0 ng) or saline for up to 72 h at 18 °C in a CO_2 incubator.

2.4. MF and ecdysteroid estimation

MF was extracted from the medium (1.0 mL) by adding 2.5 mL of CH_3CN and 0.5 mL of 4% NaCl (w/v), centrifuged at 1000×*g* for 10 min at 4 °C, and partitioned against 1 mL of hexane. The dried hexane extract was used for the determination of MF using HPLC. To determine circulating MF levels, freshly drawn hemolymph samples (1.0 mL) were mixed with 2.5 mL of acetonitrile and 1.0 mL of 1% NaCl, and then extracted with hexane using the procedures described previously. In brief, samples containing MF were separated using a bondapak C18 column (Waters, Miliford, MA, USA) with an isocratic elution of 70% acetonitrile in water (1.0 mL). Peaks were detected at 254 nm by UV absorption. Retention times of the peaks were compared to those of standard MF. The MF was also confirmed by co-chromatography with authentic all-trans-methyl farnesoate (gift from Dr. Ramachandra Reddy, Department of Biochemistry, Yogi Vemana University, Y.S.R. Kadapa District, Andhra Pradesh, India). The recovery of extracted MF from culture medium or hemolymph was 95 ± 3%. MF levels were expressed as ng/mL and ng/MO.

After 72 h incubation, 50 μL of the medium was removed from each well and extracted in 75% methanol and centrifuged (4000×*g*, 10 min). The supernatant was removed and the pellet was washed again with 75% methanol and centrifuged. The methanolic supernatants were combined and stored at −20 °C. The radioimmunoassay was conducted as previously described (Chang and O'Connor, 1979) using the ecdysteroid antiserum (a generous gift from Dr. W.E. Bollenbacher, University of North Carolina, Chapel Hill, USA). The sensitivity of the assay was calculated as 0.3 ng and intra-assay variation was found to be 6.2%. All of the samples were run at the same time to avoid inter-assay variation. Ecdysteroid concentrations were expressed as ng/mL and ng/YO.

2.5. Hemolymph collection and tissue isolation

Hemolymph was collected by withdrawing from the sinuses at the base of the third walking leg with a syringe and mixed (1:2) with anticoagulant (containing 1.4% Na_2HPO_4, 1.3% KH_2PO_4, 0.3% ethylene diamine tetra acetic acid (EDTA), 2% dextrose, and 0.25% sodium citrate). The hemolymph samples were stored at −40 °C, after centrifugation at 4000×*g* for 10 min at 4 °C. Hepatopancreas