Molecular mechanism of molt-inhibiting hormone (MIH) induced suppression of ecdysteroidogenesis in the Y-organ of mud crab: Scylla serrata

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Abstract The present study was focused on the regulation of ecdysteroidogenesis in the Y-organ of Scylla serrata during molting cycle. A strong expression of molt-inhibiting hormone (MIH) and phosphorylation of ERK was predominantly observed in the postmolt and intermolt stages of Y-organs, whereas protein kinase C, steroidogenic acute regulatory protein (StAR) and cytochrome P450 secretary activity were exclusively seen in the premolt stages. Interestingly, inhibition of ERK phosphorylation by PD98059 in the early postmolt (A), middle postmolt (B) and intermolt (C) stages resulted in the prominent expression of PKC and StAR in the postmolt stages. This result indicates that phosphorylation of ERK is required for suppression of ecdysteroidogenesis with the involvement of protein kinase C and StAR protein. © 2007 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

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1. Introduction

Molting is controlled by the ecdysteroids and molt-inhibiting hormone (MIH) secreted by Y-organs, X-organs and Sinus gland (XO–SG) complex, respectively. Expression of MIH is under the control of ERK/MAPK pathway and inhibition of phosphorylation of ERK accelerates the molting process [1]. The inhibitory action of MIH on ecdysteroid synthesis is closely related to the molt cycle and is stage specific.

Ecdysteroid exist predominant in two forms, the inactive parent compound ec dysone and its biologically active metabolite, 20 hydroxyec dysone (20E), which is synthesized by the action of monoxygenase in peripheral tissues [2–4]. Cytochrome P450sec of inner mitochondrial membrane protein catalyzes the conversion of cholesterol to the first steroid, pregnenolone [5,6]. However, free cholesterol needs to be translocated across the outer membrane to the inner membrane of mitochondria for steroid synthesis [7]. Transfer of cholesterol into the mitochondria is facilitated by a novel steroidogenic acute regulatory (StAR) protein [8,9].

Since StAR protein mediates the rate-limiting steps in steroidogenesis, its expression must be finely regulated in the steroidogenic cells at the appropriate developmental stage. Furthermore, StAR gene expression must be able to respond rapidly to various signals that regulate steroidogenesis. Therefore, the aim of the present study was to understand the role of PKC and StAR proteins in the MIH inhibitory pathway of ecdysteroid biosynthesis in the different molting stages of Scylla serrata.

2. Materials and methods

2.1. Animal maintenance

The mud crab (S. serrata) was purchased directly from coastal regions of Muttukadu and Kovalam near Chennai, India. They were maintained in the laboratory in plastic containers at ambient temperature. The crabs were fed with fresh (or) frozen shrimp and Tilapia fish on alternate days.

2.2. Observation of molting stages

The maxilliped exopodite of S. serrata is sufficiently broad and transparent to allow precise observation of the molt related events by means of setal development and is used in the present study as a tool to accurately assess the molt stages. The III maxilliped was carefully removed using a pair of scissors, placed on a clean glass slide, and microscopically observed for the molt related events in setal development.

2.3. Immunohistochemistry

Eyestalk and Y-organ were dissected from S. serrata. Tissues were fixed in Bouin’s fixative for 24 h, serially dehydrated in alcohol and embedded in paraffin. Wax sections of 10 μm thickness were collected over clean slides and bathed for 30 min at 50 °C. The slides were deparaflinized in xylene, hydrated in alcohol and immersed in citrate buffer. They were blocked using 3% BSA in PBS for 1 h at room temperature. The sections were then incubated overnight at 4°C with specific primary antibodies for MIH (1:300) (gifted by Dr. Tsuyoshi Ohira, University of Tokyo, Japan), StAR (1:400) (Santa Cruz Biotechnology) and apoB (1:400). The slides were washed with 1·PBS, 1·PBS in a dark room for 10 min. The slides were deparaflinized in xylene, hydrated in alcohol and immersed in citrate buffer. They were blocked using 3% BSA in PBS for 1 h at room temperature. The sections were then incubated overnight at 4°C with specific primary antibodies for MIH (1:300) (gifted by Dr. Tsuyoshi Ohira, University of Tokyo, Japan), StAR (1:400) (Santa Cruz Biotechnology) and apoB (1:400). The slides were washed with 1× PBS for 5 min after which, they were incubated with the corresponding secondary antibodies conjugated with HRP in 1× PBS and 0.05% Tween 20 at room temperature. Then, slides were developed with DAB solution containing 0.05% DAB, 10 μl H2O2 in 1× PBS in a dark room for 10 min at room temperature and counter stained with haematoxylin. Finally, the sections were dehydrated and mounted with DPX. For immunofluorescence, the slides were incubated with FITC conjugated immuno globulin (1:600) for 1 h. The slides were washed in PBS and mounted in buffered glycerin.

2.4. Treatment of ERK inhibitors

The S. serrata were purchased in a uniform carapace size and weights and sorted out into different molting stages. Each group contained 5–10 crabs, of which early post molt, middle post molt, and late post molt crabs were used for treatment groups.

Since StAR protein mediates the rate-limiting steps in steroidogenesis, its expression must be finely regulated in the steroidogenic cells at the appropriate developmental stage. Furthermore, StAR gene expression must be able to respond rapidly to various signals that regulate steroidogenesis. Therefore, the aim of the present study was to understand the role of PKC and StAR proteins in the MIH inhibitory pathway of ecdysteroid biosynthesis in the different molting stages of Scylla serrata.
intermolt and early premolt stage animals were taken for inhibitor injection PD98059 (a highly selective in vivo inhibitors of ERK kinase cascade). A animals of uniform weight (300 g) were selected for the experiments. Each animal was injected with 100 µl of 30 µM PD98059 containing 1.336 µg of the ERK inhibitor PD98059 in DMSO. A 50 µM solution was diluted in dimethyl sulfoxide and injected into test crabs via the adrothal joints. The control groups were injected with dimethyl sulfoxide only. Injected crabs were maintained for 48 h, then Y-organs were dissected and homogenized in lysis buffer.

2.5. SDS-PAGE and Western blotting

X-organ-sinus glands and Y-organs of S. serrata were homogenized in 135 mM NaCl, 20 mM Tris, 2 mM EDTA and 1 mM PMSF; pH 7.4 (pH 7.4); the volume of homogenization buffer was 1 ml per 100 mg X-organ sinus gland and Y-organs tissues. The homogenates were centrifuged (15 min, 10,000 rpm at 4 °C) and the protein content of the supernatant determined according to the Lowry's method with BSA as standard. Aliquots of supernatant (20 µg total protein) were boiled for 5 min in sample buffer (0.2 M Tris–HCl buffer, 10% glycerol, 2% SDS, 0.02% β-mercaptoethanol). Proteins were separated by Tris–glycine–SDS discontinuous 10% polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membrane. After protein transfer, the membrane was incubated for 2 h at room temperature in blocking buffer (TBS pH 7.5 containing 0.05% Tween 20, 5% non-fat dried milk). After blocking, the membrane was rinsed for 5 min with washing buffer (TBS containing 0.05% Tween 20), then incubated for 16 h at 4 °C with primary antiserum diluted with TBS containing 0.05% Tween 20 and 0.3% BSA. The following antibodies containing 0.05%, Tween 20. The membrane was incubated with the respective secondary antibodies for 1 h at room temperature. Specific staining of the supernatant determined according to the Lowry’s method with BSA as standard. Aliquots of supernatant (20 µg total protein) were boiled for 5 min in sample buffer (0.2 M Tris–HCl buffer, 10% glycerol, 2% SDS, 0.02% β-mercaptoethanol). Proteins were separated by Tris–glycine–SDS discontinuous 10% polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membrane. After protein transfer, the membrane was incubated for 2 h at room temperature in blocking buffer (TBS pH 7.5 containing 0.05% Tween 20, 5% non-fat dried milk). After blocking, the membrane was rinsed for 5 min with washing buffer (TBS containing 0.05% Tween 20), then incubated for 16 h at 4 °C with primary antiserum diluted with TBS containing 0.05% Tween 20 and 0.3% BSA. The following antibodies were used: MIH (1:8000), StAR (1:10000), phospho ERK (1:10000) (Santa Cruz Biotechnology). After an over night incubation with primary antibody, the membrane was washed 3× (5 min each) with TBST containing 0.05%, Tween 20. The membrane was incubated with the respective secondary antibodies for 1 h at room temperature. Specific signals were detected by chemiluminescence using luminogen substrate (Amersham, ECL advance, Western blotting Detection Kit). Enchanced chemiluminescence (ECL) signals were recorded on X-ray film.

2.6. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNA was extracted by Trizol Kit according to the manufacturers instructions. The total RNA was preheated for 5 min at 70 °C, immediately cooled on ice, and reverse-transcribed for 50 min at 42°C in a 20 µl reaction mixture containing 10 mM dNTP’s, 10 mM Oligodeoxynucleotides, 25 units of reverse transcriptase and 2 µl of assay buffer. The RT reaction was terminated by heating for 5 min at 70 °C. The resulting cDNA templates were subjected to PCR amplification. Each cycle consisted of 30 s at 95 °C for annealing and 1 min at 72 °C for extension. The reaction mixture contained 2 mM of dNTP’s, 2U of Taq polymerase, 2 µl of cDNA. The cytochrome P450c6, primers-Sense-5′-AACGTCCTCCAGAAGCTCCA-GAACCTGTACC-3′, antiseense-5′-CTTGCTTATGTCTCCCTTGC-GCC-3′), were synthesized according to the cDNA sequence cloned by Morohasi et al [11]. The PCR reaction products were electrophoresed on 2% agarose gels with ethidium bromide and visualized in UV light (Gel documentation, Amersham Ltd).

3. Results

3.1. MIH in X-organ-sinus gland complex and Y-organ cells

The immunohistochemical analysis of eyestalk neural ganglia shows a highly positive immunoreactivity of MIH in the X-organ and sinus gland complex of the eyestalk of S. serrata only in the post molt (A and B) and intermolt (C) stages using anti-pej-MIH (Fig. 1b). There was no expression of MIH observed in negative control (without primary antibody) in intermolt (C) (Fig. 1a).

The membranous expression of MIH protein was observed in all molting stages of S. serrata in Y-organ tissues. The high intensity of their localization was seen in postmolt (A and B) and intermolt (C) stages, but lowest level of distribution was observed in the premolt stages (D0, D1 and D2,3) (Fig. 1c).

Fig. 1. (a) A control section of eyestalk, no immunoreactivity of MIH was observed. (b) Represents whole structure of eyestalk, showing three distinct structures of medulla externa, medulla interna and medulla terminals. Medulla interna and terminals showing higher immunopositive reaction of MIH protein. (c) The distribution of MIH protein on the target cell of Y-organs. Membranous expression of MIH protein on the Y-organ cells is seen (red arrow). (d) Shows the membranous expression of ApoB in the premolt stage of Y-organ cells. Membranous expression as well as cytosolic expression indicates that ApoB predominantly accumulated in the cytosolic region for ecdysteroid biosynthesis. red arrow indicates the counter stain with haematoxylin (blue). (e) Expression of StAR protein in the premolt stage of Y-organs cells. Results shows that cytosolic distribution of StAR is protein required for delivery of cholesterol from outer membrane to inner membrane of mitochondria. (f) Represents Oil Red 'O' staining of cholesterol in the Y-organ cells. cholesterol is predominantly distributed in the cytosolic region of Y-organ of premolt stages.
3.2. Immunoblot analysis of MIH protein in the X-organ sinus complex

The protein extract was prepared from X-organ sinus gland complex of the eyestalk of *S. serrata* during different molting stages and analysed by Western blotting. Approximately 7–8 kDa of MIH protein was continuously expressed in all molting stages except in early premolt (D0) and middle premolt stage (D2–3). But intensity of its expression varied in each stage, the highest intensity seen only in intermolt stage (C) (Fig. 2b).

3.3. Expression of phospho ERK in Y-organ

The Y-organs were dissected out from the cephalothoracic region of *S. serrata* and expression pattern of phospho ERK was identified by using anti phospho ERK antibody. ERK phosphorylation was seen only in the early post molt (A), middle postmolt (B) and intermolt (C) stages, but not in the early premolt (D0), middle premolt (D1) and late premolt (D2–3) stages (Fig. 2c).

3.4. Expression of protein kinase C in Y-organ

The expression of protein kinase C was observed only in the early premolt (D0, D1 and D2–3) stages using anti protein kinase C antibody but not in the postmolt (A and B) and intermolt (C) stages (Fig. 2d).

3.5. Distribution of apoB and Cholesterol in Y-organ cells

The Y-organ directly depends on circulatory cholesterol for ecdysteroid biosynthesis. Crustaceans cannot synthesize the cholesterol and Y-organ does not store it, hence the animal directly depends on dietary cholesterol for ecdysteroid biosynthesis. The apo B part of LDL was predominantly observed only in early premolt (D0), middle premolt (D1) and late premolt (D2–3) stages. In the early premolt stage (D0) apoB was seen in the cytosolic region (Fig. 1d), whereas, in the middle and late premolt stages (D1 and D2–3) apoB was intensively accumulated in the cytosolic region (Fig. 1d).

Cholesterol is a major precursor molecule for ecdysteroid biosynthesis in the Y-organ cells (Steroidogenic cells). In this study, cholesterol has been observed in all molting stages of the Y-organ cells, but high content of cholesterol was predominantly accumulated in the Y-organ cells in premolt stages such as early premolt (D0), middle premolt (D1) and late premolt (D2–3) stages (Fig. 1e).

3.6. Expression of StAR protein

The steroidogenic acute regulatory (StAR) protein regulates the rate limiting steps of steroid biosynthesis. StAR is predominantly associated with steroid producing tissues and consists of a 37 kDa precursor form containing an N-terminal mitochondrial targeting sequence and several isoforms of a 30 kDa mature protein. In this study, the stage specific expression of the 30 kDa StAR protein was found only in the early premolt (D0), middle premolt (D1) and late premolt (D2–3) stages. Among these stages, phosphorylation of StAR protein was seen only in the early premolt and middle premolt stages (Fig. 2e). Immunolocalization of StAR protein was also done in Y-organ cells, cytosolic distribution of StAR protein was predominantly observed in early premolt (D0, D1 and D2–3) (Fig. 1e).

3.7. Effect of PD98059 (ERK inhibitor) on the expression of PKC and StAR

Usually protein kinase C in the Y-organ is not expressed in postmolt (A and B) and intermolt stages (C) and ERK was always observed to be phosphorylated during these stages. When PD98059 is injected, ERK phosphorylation was blocked in these stages (Fig. 3a) and protein kinase C was expressed (Fig. 3b), which clearly shows that protein kinase C is under the control ERK phosphorylation. Similarly, StAR protein is not expressed in middle postmolt (B) and intermolt stages (C). While inhibiting the phosphorylation of ERK in these stages, StAR protein is expressed in the postmolt stages (A and B) (Fig. 3c).

3.8. Cytochrome P450 enzyme mRNA in Y-organ

The process of steroid biosynthesis is acutely regulated by mobilization of cholesterol to inner mitochondrial membrane and chronically by upregulation of many genes that express steroidogenic enzymes such as cytochrome P450, that catalyze the conversion of cholesterol to first steroid pregnelone. In this study, the mRNA level of cytochrome P450 enzyme was analysed in the Y-organ cells. The abundance of cytochrome P450 mRNA expression was seen in the post molt (A and B) and intermolt (C) stages (Fig. 4b).
4. Discussion

Although we observed a near consistent expression of MIH throughout the molting cycle, it is apparent that the intensity of MIH varies significantly and showed the maximum expression in the middle post molt (B) and intermolt (C) stages. Such a stage specific variation in the MIH titre reflects more on the ecdysteroid production and determines the responsiveness of the Y-organ. Studies have indicated that there exists no change in the number of MIH receptors in the Y-organ during molt cycle in the *Carcinus maenas* and that the Y-organ is preferentially inactivated at the intermolt stages [12], and ceases to produce ecdysteroid. MIH, though localized on the plasma membrane of steroidogenic cells in all molting stages, is predominantly distributed during the postmolt and intermolt stages in the Y-organ cells. This suggests a signaling mechanism that suppresses ecdysteroid production by regulating the transcription and translation of steroidogenic enzymes. Recent reports also suggest a relation between the rate of ecdysteroid production and the responsiveness of the Y-organ to MIH, but the cellular mechanism that underlies these changes is not well understood [12,13].

In the study, ERK is phosphorylated only in the early post-molt, middle post molt and intermolt stages but no phosphorylation was seen in premolt stages of the Y-organ cells. The stage specific phosphorylation of ERK suggests that MIH predominantly binds its receptor on Y-organ cells and suppresses the ecdysteroid biosynthesis via phosphorylation of ERK in order to activate the specific transcription factor for inhibition of steroidogenic enzymes. Previous results demonstrated that Chelerythrine C, an inhibitor of PKC, could block ecdysteroid biosynthesis in *Bombryx mori*, indicating that PKC might indeed participate in PTTH-based signaling in the prothoracic gland [14]. The activation of protein kinase C pathway results in the transduction of signals that are able to increase the transcription and translation of StAR gene [15]. We also observed a similar result that, protein kinase C (PKC) was seen only in the early premolt, middle and late premolt stages, but not in the postmolt stages. PKC undergoes a series of phosphorylations, including auto and trans-phosphorylations that render it competent to interact with target proteins. Modulation of ecdysteroidogenesis by PKC has been reported in (tobacco hornworms) *Manduca sexta* [16]. However, in contrast to insects, crustaceans have two mechanisms, one involving inhibition of ecdysteroidogenesis and the other activation of ecdysteroidogenesis by PKC, which is independent of MIH pathway. Indeed, despite the presence of MIH in the premolt stages, Y-organs do synthesize ecdysteroids through signaling
mechanism that overrides the MIH pathway [12]. In this study, StAR proteins are found to be expressed only in the premolt stages. Previous results suggest that, StAR protein appears to be involved in the transfer of cholesterol from the outer to the inner mitochondrial membrane, which is the rate-limiting and regulatory step in steroidogenesis.

StAR is predominantly associated with steroid production and consists of a 37 kDa precursor form containing an N-terminal mitochondrial targeting sequence [17,18]. StAR protein expression significantly increases through the activation of PKC pathway. In addition, ligand interaction with receptors can activate phospholipase C and trigger the activation of the downstream PKC pathway [19–21].

In this study, during the early pre molt stage, apoB was localized on the plasma membrane of the Y-organ, whereas, in the middle and late premolt stages apoB begins to penetrate into the cytosolic region and accumulates in large amounts for cholesterol side chain cleaving process. This result indicates that all the circulating cholesterol is bound to low-density lipoprotein (LDL) and also has a high binding capacity to Y-organ. These results indicate that accumulation of large amount cholesterol in this stage is mainly for ecdysteroid biosynthesis. The Y-organ directly depends on circulatory cholesterol for ecdysteroid biosynthesis. Crustaceans cannot synthesize cholesterol and Y-organ does not store it, Hence the animal directly depends on dietary cholesterol for ecdysteroid biosynthesis.

Cytochrome P450_20sec is an inner mitochondrial membrane protein that catalyzes the conversion of cholesterol to the first steroid, pregnenolone [4,5]. For this purpose, free cholesterol needs to be translocated across the outer membrane to become available as substrate at the site of P450_20sec [6]. In this study cytochrome P450_20sec transcript was seen only in the premolt stage and among premolt stage and the early premolt stages has higher intensity of cytochrome P450_20sec mRNA, clearly indicating that StAR protein is required for cholesterol transport from the outer to the inner mitochondrial membrane site of the cytochrome P450_20sec enzyme for ecdysteroid biosynthesis.

This pathway is confirmed by the inhibition of phospho ERK by using PD98059 in the early postmolt (A), middle postmolt (B) and intermolt (C) stages, the results showing that in the absence of phospho ERK, protein kinase C is predominantly expressed in the post molt and intermolt stages. This indicates that phosphorylation of ERK could suppress the PKC mediated pathway, which is required for activation of StAR protein for ecdysteroid biosynthesis. MIH binds to plasma membrane of Y-organ and suppresses the ecdysteroid biosynthesis in the postmolt and intermolt stages via phosphorylation of ERK. Since phospho ERK is absent, PKC is expressed in post molt and intermolt stages. We suggest that, MIH induced ERK activation through Ras/Raf pathway could inhibit the transcription of StAR gene by activation of DAX-1 transcription factor, which is a member of nuclear hormone receptor family. It has been shown that DAX-1 binds to hairpin loop structures and suppresses transcription [22], whereas activation of PKC in the premolt stages can regulate transcription factors CREB, SP-1, SF-1 for transcription of StAR gene (see Fig. 5).

Therefore, we conclude that MIH induced suppression of ecdysteroid production by phosphorylation of ERK and the presence of protein kinase C, apoB, StAR protein and cytochrome P450_20sec enzyme in the premolt stages might have a spe-

Fig. 5. MIH induced ERK activation via Ras, Raf pathway involving the suppression of ecdysteroidogenesis and PKC mediated StAR transcription.
cific role in ecdysteroid biosynthesis. Future studies will expand our understanding on protein kinase C activated ligand for ecdysteroid biosynthesis.

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