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Expression Analysis of Ecdysone Receptor and Ultraspiracle through Molting Period in Mysid Crustacean, *Americamysis bahia*

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Abstract—Ecdysteroids, primarily 20-hydroxyecdysone (20E), play a central role in the growth of arthropods. Molecular target of the ecdysteroids is known as a receptor complex composed of ecdysone receptor (EcR) and ultraspiracle (USP) protein. It is important to clarify the expression profile of EcR/USP in crustacean for understanding the endocrine disrupting effects of environmental xenobiotics on crustacean. However, despite EcR/USP has been characterized in insect, little is known about their complex functions in crustacean species. In this study, in order to gain better understanding of the mechanism of molting in mysid (*Americamysis bahia*), the molting stage-specific expressions of EcR/USP were examined. The semi-quantitative RT-PCR analysis revealed that the expression level of the EcR increased during inter- and pre-molt stages (from 36 to 48 h after molting). This expression pattern correlated with the time of increasing titers of 20E, suggesting the essential role of EcR in the timing of molt. In the *A. bahia* exposed to 20E and nonylphenol for 8 h, the expression levels of EcR/USP mRNA were up-regulated when compared with controls. These results suggest that EcR/USP gene expression were associated with molting control in *A. bahia*, and may have been transcriptionally altered by exposure to nonylphenol.

Keywords: mysid, *Americamysis bahia*, molting, ecdysone receptor, nonylphenol

INTRODUCTION

Ecotoxicological impact of environmental xenobiotics not only on vertebrates but also on invertebrates is currently a worldwide concern, especially in terms of the impact of pollution on whole ecosystems. However, studies on the specific mechanism by which the endocrine system of invertebrate can be disrupted are scarce; this is a critical point to be developed for fully understanding the risk of known endocrine-disrupting chemicals (EDCs) (DeFur *et al*., 1999; Rodríguez *et al*., 2007).

Ecdysteroids, primarily 20-hydroxyecdysone (20E), play crucial roles in arthropods by initiating and regulating molting and metamorphosis. Besides their functional role as molting hormone, ecdysteroids are involved in the control of reproduction and embryogenesis (Subramoniam, 2000). Molecular target of the ecdysteroids is known as an ecdysone receptor (EcR), which belongs to the nuclear receptor family. EcR is a ligand-dependent transcription factor and it activates transcription of target genes by forming a heterodimer with another member of the nuclear receptor family, ultraspiracle protein (USP), which is the insect homologue of vertebrate retinoid X receptor (RXR) (Yao *et al*., 1992).

In insects, EcR has been well characterized, especially in fruit fly *Drosophila melanogaster* and silkworm *Bombyx mori* (reviewed by Thummel, 1995; Kamimura *et al*., 1996, 1997). In contrast, our understanding of the characteristics of crustacean EcR and RXR at the molecular level remains limited. To date, EcR and/or RXR have been fully sequenced in only some crustacean species, including decapod crustaceans (*Celuca pugilator*, Durica and Hopkins, 1996; Chung *et al*., 1998a; *Gecarcinus lateralis*, Kim *et al*., 2005; *Marsupenaeus japonicus*, Asazuma *et al*., 2007), water flea *Daphnia magna* (Wang *et al*., 2007; Kato *et al*., 2007) and mysid crustacean *Americamysis bahia* (Yokota *et al*., 2005). These studies on gene expression analysis indicate that the crustacean EcR/RXR complex functions as a mediator of ecdysteroid signals as well as in insects, although the expressions regulated the different expression pattern of isoforms in a tissue-specific manner. Thus, it is important to clarify the expression profile of EcR and USP genes in each crustacean species for understanding of hormonal systems in crustacean. Understanding of EcR and USP is also important from the ecotoxicological point of view. The possibilities of the adverse effects of environmental chemicals on crustacean molting had been investigated in several earlier studies (DeFur *et al*., 1999; Zou, 2005). However, studies about its disruption by chemicals through specific hormone-regulated mechanisms remain extremely limited.

Among aquatic invertebrate species, mysid crustacean *A. bahia* have been used in toxicity testing for more than two decades, because of their ecological relevance and with the idea they can serve as a model for the responses of many other crustaceans (reviewed by Verslycke *et al*., 2004, 2007). Despite the fact that mysids have been put forward as suitable test organisms for assessing EDCs by several agencies, including the US Environmental Protection Agency (USEPA), the American Society for Testing of Materials (ASTM), and Organization for Economic Cooperation and Development (OECD) (reviewed by Verslycke *et al*., 2004, 2007), our understanding of hormone regulation and its disruption by EDCs is limited. As described above, recently, Yokota *et al*. (2005) isolated the entire sequence of cDNA encoding EcR and USP from *A. bahia*, and developed an *in vitro* binding assay which holds promise as a rapid screen of chemical interaction with the mysid ecdysteroid receptor complex. However, there is no information on expression analysis of mysid EcR and USP genes *in vivo*. Our previous study has demonstrated that the decrease and/or delay of peak 20E levels in mysid crustacean exposed to xeno-estrogen nonylphenol (a major degradative product of nonylphenol ethoxylates which are widely used as nonionic surfactants) were

associated with adverse effects on the development that were linked to delays in molting cycles (Hirano *et al.*, 2008). These findings imply the possible involvement of EcR and USP in the molt-inhibiting effects of nonylphenol. In this study, therefore, in order to gain better understanding of the mechanism of molting in mysid *A. bahia*, the molting stage-specific expressions of EcR/USP mRNA were examined. Furthermore, we investigated expression levels of EcR/USP mRNA in mysid exposed to 20E and nonylphenol.

MATERIALS AND METHODS

Test chemical

20E (ICN Biomedicals Inc., Ohio, USA) and nonylphenol (Aldrich Chemical Company Inc., Tokyo, Japan) were used in this study. Test solutions were prepared by dissolving the reagents in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., Tokyo, Japan).

Test organisms

Mysids (Chesapeake Cultures, Inc., Virginia, USA) were originally obtained from the Japan Pulp and Paper Research Institute, Inc. (Tsukuba, Japan). The culture originated at the USEPA (Gulf Breeze, Florida, USA). A breeding stock of these mysids has been maintained in a 25-l glass tanks under a 16:8 light:dark photoperiod at 25 ± 1°C (the pH and salinity were 8.0 and 25‰, respectively) in our laboratory. These mysids were fed daily with *Artemia* nauplii (<24 h after hatching) ad libitum.

Temporal expression of EcR/USP mRNA through molt period

For gene expression analysis in a molt period, adult female mysids were used. Gravid females of approximately the same size (body weight) were selected and individually distributed to 200 ml glass beakers along with 150 ml of artificial seawater with a salinity of 25‰. The presence of exuviae was checked each morning, and three newly-molted mysids each were randomly put in 300 ml glass beakers containing 250 ml of the test solution. As described above, the time of molting was assigned as 0 h and samples were collected every 12 h for the following 96 h. The solutions were renewed every 24 h. Test organisms were fed a diet of approximately one hundred fifty *Artemia* nauplii (<24 h after hatching) per mysid per day. Mysids were washed briefly and immediately frozen in liquid nitrogen, and then stored at –80°C until analysis.

Exposure design of test compounds

Changes in EcR and USP mRNA levels in response to 20E, or nonylphenol in adult female mysids were evaluated. As described above, newly-molted mysids were exposed to 1000 μg/l 20E, 30 μg/l nonylphenol, or carrier solvent (controls, 0.01% DMSO) for 8 h. The selected range of concentrations for the test

was based on the results of the 96 h acute toxicity test performed previously by Hirano *et al*. (2004). Three newly-molted mysids each were randomly put in 300 ml glass beakers containing 250 ml of the test solution. All individual mysids were analyzed for EcR and USP mRNA levels. The mysids were subjected to expose for 8 h under the light photoperiod, and were not fed during the exposure periods. At the end of the 8 h exposure, mysids were washed briefly and immediately frozen in liquid nitrogen, and then stored at –80°C until analysis.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the frozen mysid by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method using TRIzol® reagent (Invitrogen Co., Ltd., Japan) according to the manufacturer's protocol. The RT reaction mixture contained 1 μg of total RNA, 1 μl of 25 pmoles random primers and diethylpyrocarbonate (DEPC)-treated water. The reaction mixture was heated to 70°C for 10 min and quickly chilled on ice. After cooling, 4 μl of 5 x reaction buffer containing 25 mM $MgCl_2$, 2 μl of deoxynucleotide triphosphates (dNTPs; 10 mM each) were added, and then were pre-incubated at 25°C for 5 min. After the incubation, 1 μl of ReverTra Ace (Toyobo, Co., Ltd., Osaka, Japan) were added to a final volume of 20 μl. Reverse transcription reactions were carried out at 30°C for 10 min and 42°C for 60 min. The reaction mixture was then heated at 95°C for 5 min to stop the RT. PCR amplifications were performed on a PCR thermal cycler. Amplification of 0.2 μl cDNA mixture was carried out in 20 μl containing TaKaRa Ex Taq polymerase (TaKaRaBio, Shiga, Japan), 10x PCR buffer (with 1 mM $MgCl_2$), 0.2 mM of each dNTP and 0.2 M of both sense and antisense primers. The primer pair sequences were designed based on *A. bahia* EcR (GenBank accession no. DD410574), USP (DD410577) and 18S rRNA (AJ566095). The total volume of the reaction mixture was 20 μl. The PCR conditions were as follows; initial denaturation step at 94°C for 5 min and subsequent denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min for 35 cycles for EcR and 18S rRNA, and 30 cycles for USP, and a final extension at 72°C for 5 min. The amplified PCR products were analyzed by electrophoresis on 1.5% agarose gels. The bands were visualized by ethidium bromide staining (15 μl of a 10 mg/ml ethidium bromide solution per 100 ml water for 10 min). The band densities of amplified products were calculated using 1D Image Analysis Software (Kodak Digital Science, Rochester, NY, USA). Values were normalized against the 18S rRNA as an internal standard and the relative expression levels were then calculated.

Statistical analysis

Significant differences in relative ratios of EcR, USP and 18S rRNA mRNA between groups were analyzed by Student's *t*-test. Difference was considered significant at $p < 0.05$. All statistical analyses were performed using SPSS 16.0J (SPSS Japan, Tokyo, Japan).

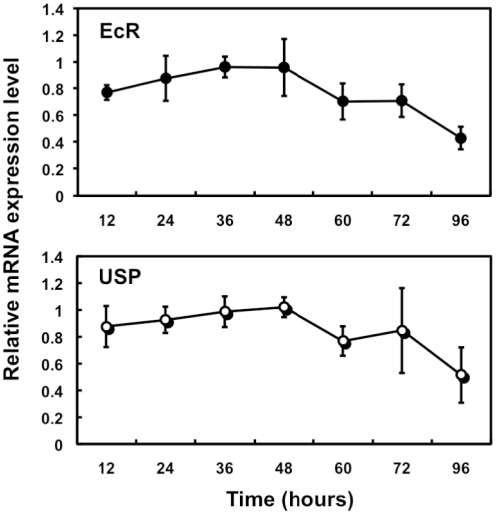

Fig. 1. Changes in expression levels of EcR and USP mRNA in *A. bahia* through a molting period. Transcription levels of EcR (●) and USP (○) in mysid were analyzed using the semi-quantitative RT-PCR (relative to 18S rRNA expression). Error bars show the standard deviation of the mean (n = 3).

RESULTS AND DISCUSSION

In the present study, the expression pattern of mysid EcR and USP mRNA was evaluated in adult female mysid through molt stage. The expression level of EcR mRNA were increased around 48 h and progressively declined through molt stage (Fig. 1). Moreover, temporal expression profile of USP mRNA was similar to that of EcR mRNA (Fig. 1). Our previous study demonstrated that the 20E production in a normal molting period of mysid reached maximum levels in 48 h (Hirano *et al*., 2008). The expression patterns of EcR and USP mRNA were correlated with elevated ecdysteroid levels, suggesting the essential role of EcR/USP in the timing of molt. In insect EcRs, the presence of multiple EcR subtypes exist in the N-terminal A/B domain caused by alternative splicing, and their expression is regulated by different promoters, resulting in the different expression pattern of isoforms in a tissue-specific manner (Bender *et al*., 1997; Shirai *et al*., 2007). In crustacean EcR, Kato *et al*. (2007) recently isolated three isoforms of EcR (one EcR-A isoform and two EcR-B isoforms) from *D. magna* that differ in the A/B domain, and indicated differing temporal expression patterns of the EcR isoforms during the molting period. Their observation of differential isoform expression revealed that although both EcR-A and EcR-B play a distinct role in molting, gene expression levels of EcR-B were more prominent than EcR-A in the whole body of *D. magna* (more than 20-fold). In decapod crustacean, transcript levels of EcR and RXR in several tissues except for hepatopancreas and/or muscle tissues were not clearly correlated with circulating ecdysteroid titers

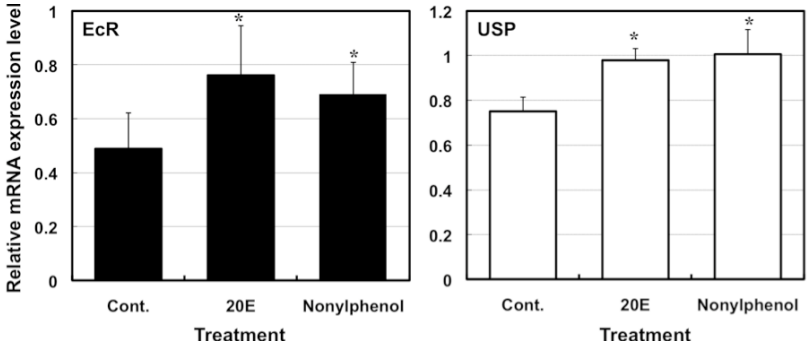

Fig. 2. Relative EcR and USP mRNA levels in adult female *A. bahia* exposed to either 20E-1000 μg/l or NP-30 μg/l for 8 h. EcR and USP mRNA levels were normalized to 18S rRNA level in each sample. Data are presented as the mean and standard deviation ($n = 3$). An asterisk denotes a significant ($p < 0.05$) difference from the control.

(Chung *et al*., 1998b; Asazuma *et al*., 2007), and this implies that the expression of these genes was not controlled by ecdysteroid only. Since our primers are designed for the common region of the receptor genes, our results represent changes of total mysid EcR and USP transcript during the molt cycle. Overall, therefore, our observation of the temporal expression patterns of mysid EcR and USP mRNA in whole body suggests that these genes play distinct roles in mysid crustacean. However, further studies are required to understand their function, not only clarification of tissue-specific expression patterns but also the existence of isoforms in mysid crustacean.

Transcriptional modulation of mysid EcR and USP mRNA levels by 20E and nonylphenol was assessed in adult female mysid (Fig. 2). EcR mRNA levels were significantly increased with regards to not only 20E but also nonylphenol exposure compared to the control. In the present study, the effect of test compounds on the expression of EcR and USP mRNA levels in newly-molted mysids was assessed. Newly-molted mysids were used to avoid possible effects of the endogenous ecdysteroid levels on EcR and USP gene expression in these mysids. EcR and USP mRNA levels were not significantly influenced by chemical treatment for 48 h (data not shown). Previously, induction of insect EcRs mRNA by ecdysteroid has been investigated *in vitro* (Hiruma *et al*., 1997; Kamimura *et al*., 1997). In *Bombyx*, especially, EcR-A and EcR-B mRNA were induced within 2 h and 1 h, respectively, of the addition of 500 μg/l 20E when the anterior silk glands of day 0 of the fifth instar were cultured (Kamimura *et al*., 1997). Mysid EcR mRNA levels in whole body were up-regulated when exposed via the water to 1000 μg/l 20E for 8 h, indicating that mysid responded rapidly to 20E. On the other hand, we also detected an increase of mysid USP mRNA. Similar results for daphnid RXR were obtained using adult female (Wang *et al*., 2007).

The present study suggests that the expression level of EcR mRNA in female mysid was induced by treatment with nonylphenol. A recent study also showed

the expression levels of EcR mRNA in the aquatic insect *Chironomus riparius* larvae could be induced by xeno-estrogen bisphenol A *in vivo* (Planelló *et al*., 2008). These findings together with our results indicate that mysid EcR are transcriptionally altered by exposure to estrogenic nonylphenol. Furthermore, Dinan *et al*. (2001) demonstrated that 4-nonylphenol is a weak antagonist compound using an ecdysteroid-responsive *Drosophila* cell based assay. Lye *et al*. (2008) also showed a significant reduction in ecdysteroid levels in male shore crabs exposed to 4-nonylphenol. In the previous study, we observed a decrease in 20E levels in mysids exposed to 30 μg/l nonylphenol (Hirano *et al*., 2008). These results suggest that nonylphenol caused reduction of 20E levels in mysid associated with change of the expression levels of EcR mRNA. Therefore, further studies are required to ascertain whether these chemicals including nonylphenol have interaction with the EcR ligand-binding site, and to understand the interplay between changes in the EcR/USP complex and other ecdysone responsive genes. Such studies should lead to mechanistic understanding of hormone regulation in mysids and its potential disruption by environmental chemicals.

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