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Short communication

The exogenous methyl farnesoate does not impact ecdysteroid signaling in the crustacean epidermis in vivo

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ABSTRACT

Methyl farnesoate (MF) produced by the mandibular organ is a crustacean terpenoid hormone involved in the regulation of larval development, reproduction and male morphogenesis. But the receptor for MF has remained unresolved. In view of the fact that MF can bind to crustacean retinoid X receptor (RXR) and that the terpenoid mimic, pyriproxyfen, is capable of altering the expression of crustacean RXR gene, crustacean RXR has been proposed to be a candidate receptor for MF. It is well known that ecdysteroids signal through the ecdysteroid receptor (EcR), which heterodimerizes with the RXR in Crustacea. This study was aimed to investigate whether the exogenous MF impacts ecdysteroid signaling in vivo using *N*-acetyl-β-glucosaminidase (NAG) mRNA from epidermal tissue as a biomarker for ecdysteroid signaling. The NAG mRNA from the model crustacean *Uca pugilator* injected with 0, 0.2, 1, 5, and 20 ng/g wet weight of MF was quantified using quantitative real-time PCR (qRT-PCR). An assay of epidermal NAG activity in crabs injected with 0, 20, and 2000 ng/g wet weight of MF was also performed. The administration of the exogenous MF was found to have no effects on epidermal NAG gene transcription or NAG activity in *U. pugilator*. These results clearly show that MF is not capable of affecting epidermal ecdysteroid signaling in the fiddler crab, *U. pugilator*. Our data are not supportive of the notion that MF signals through the RXR in Crustacea.

1. Introduction

Methyl farnesoate (MF) is a crustacean terpenoid synthesized and secreted from the mandibular organ, a homologue of the insect corpora allata (Nagaraju, 2007). The production of MF by the mandibular organ is negatively controlled by the mandibular organ inhibitory hormones produced in the X-organ sinus gland complex in the eyestalk (Landau et al., 1989; Laufer et al., 1987). Insects contain a group of terpenoid compounds, known as juvenile hormones, which regulate metamorphosis and gametogenesis (Laufer et al., 1987). MF is the unepoxidated form of the insect juvenile hormone III (JHIII) (Abdu et al., 1998), and has been shown to exhibit insect JH-like activities in crustaceans. Larvae of the lobster, *Homarus americanus*, showed an increase in time to metamorphosis when exposed to the exogenous MF (Borst et al., 1987). In the freshwater prawn, *Machrobrachium rosenbergii*, exogenous MF resulted in the delay of larval development and morphogenesis and caused an increase in intermediate specimens (Abdu et al., 1998). MF has also been implicated in the regulation of crustacean reproduction, capable of stimulating ovarian maturation and testicular development. An increase in the hemolymph titer of MF was found to coincide with ovarian development in *Libinia emarginata* (Laufer et al., 1987), and injection of the exogenous MF stimulated ovarian maturation and testicular development in *Oziotelphusa senex* (Reddy et al., 2004). Recently, MF has been found to be a crustacean morphogen, involved in controlling adult male morphogenesis in *Procambarus clarkii* (Laufer et al., 2005) and male differentiation in *Daphnia magna* (Olmstead and LeBlanc, 2002). As a result of the endogenous production of MF as well as the evidence of its involvement in larval development, reproduction, and male morphogenesis, MF has been recognized as a genuine crustacean hormone (Laufer and Biggers, 2001; Nagaraju, 2007).

Despite voluminous evidence of MF's hormonal roles in Crustacea, its receptor has not been definitively recognized. In view of the fact that MF can have high affinity binding with the *Drosophila* ultraspiracle (USP), a vertebrate retinoid X receptor (RXR) ortholog (Jones et al., 2006), and that the juvenile hormone mimic pyriproxyfen can alter the expression level of the RXR mRNA in *D. magna*, RXR has been suggested as a candidate receptor for MF in Crustacea (Wang et al., 2007). This suggestion seems to be supported by the results of a receptor binding study. Hopkins et al. (2008) showed that MF can not only bind to the *Uca pugilator* RXR (UpRXR) with high affinity but also influence the binding to an ecdysteroid of the *U. pugilator* ecdysteroid receptor (UpEcR)/UpRXR dimer. But the results of a recent study utilizing luciferase reporter assays run counter to the notion that MF acts through RXR since MF, but not 9-*cis* retinoic acid which is the natural ligand for RXR, failed to transactivate the reporter gene in

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human hepatocellular carcinoma cells (HepG2) transfected with daphnid RXR construct (Wang and LeBlanc, 2009).

It is known that at the cellular level crustacean ecdysteroids signal through binding to the ecdysteroid receptor (EcR), which then heterodimerizes with the RXR (Chung et al., 1998; Durica and Hopkins, 1996). The EcR/RXR heterodimer binds to the DNA response elements of the genes regulated by the molting hormone. Among the products of the genes regulated by ecdysteroids are hydrolytic enzymes necessary for the deconstruction of the old exoskeleton. *N*-Acetyl-β-glucosaminidase (NAG), a chitinolytic enzyme found in the epidermis, is involved in degradation of exoskeletal chitin. There is plenty evidence that the genes encoding epidermal chitinolytic enzymes, including chitinase and NAG, are under control of the molting hormone in arthropods. Injection of 20-hydroxyecdysone significantly increased the enzymatic activity and mRNA of both chitinase and NAG in the epidermis of the tobacco hornworm, *Manduca sexta* (Fukamizo and Kramer, 1987; Kramer et al., 1993; Zen et al., 1996). In crustaceans NAG activity has been found to be correlated with the hemolymph titer of ecdysteroids in *U. pugilator* (Zou and Fingerman, 1999a). Injection of the molting hormone stimulated chitinolytic activity in the integument of the barnacle *Balanus amphitrite* (Freeman, 1980) and elevated the activity of both chitinolytic enzymes in the epidermis of *U. pugilator* (Zou and Bonvillain, 2004; Zou and Fingerman, 1999b). Recent gene expression studies have shown that the administration of 20-hydroxyecdysone significantly upregulated the transcription of NAG gene in the epidermis of *U. pugilator* (Meng and Zou, 2009a,b). Clearly, epidermal chitinolytic enzymes are the products of the genes regulated by the molting hormone in arthropods. The expression of NAG gene in the epidermis of *U. pugilator* constitutes the terminal event of the actions of ecdysteroids, and epidermal NAG mRNA represents a biomarker for ecdysteroid signaling in the epidermis. Therefore, ecdysteroid signaling as measured by NAG mRNA in the epidermis of the fiddler crab is an excellent model for studying the signaling of ecdysteroids as well as MF, a putative ligand for crustacean RXR. The present study was designed to test the notion that MF acts through the RXR using *U. pugilator* as the model crustacean. Our reasoning was that should MF signal through the RXR, the ecdysteroid signaling in the epidermis of the fiddler crab would be impacted after administration of the exogenous MF. Our emphasis was placed on two lines of in vivo evidence, the transcription of NAG gene and the activity of NAG.

2. Materials and methods

Female fiddler crabs were purchased from the Gulf Specimen Marine Laboratories (Panacea, FL). The crabs were distributed into tanks containing artificial seawater made with Instant Ocean synthetic sea salt (Aquarium Systems, Mentor, OH). The animals were maintained under the natural light regime and at a temperature of 19–21 °C. The animals were allowed to acclimate to laboratory conditions at least 5 days before use in an experiment. Since NAG activity in the epidermis varies during the molting cycle (Zou and Fingerman, 1999a), only intermolt crabs were used in the experiment. Molt-staging was performed using the setogenic technique which is based on epidermal retraction and setogenesis in pleopods of female crabs (Vigh and Fingerman, 1985). Since in male crabs the pleopods are modified into copulatory appendages, which makes molt-staging impossible, male crabs were not used.

Quantitative real-time PCR (qRT-PCR) was used to quantify epidermal NAG mRNA from *U. pugilator*. RNeasy mini kit (Qiagen, Valencia, CA) was used to extract the total RNA from epidermal tissues followed by DNase digestion of RNA and RNA cleanup using protocols described in the RNeasy Mini Handbook. RNA concentration was determined by absorption spectrophotometry at a wavelength of 260 nm. Quality of isolated RNA was determined by the ratio of absorbance at 260–280 nm. First strand cDNA was synthesized using the Qiagen QuantiTech Reverse Transcription Kit (Qiagen, Valencia, CA). Two pairs of primers were designed to obtain a PCR product of about 150 base pairs. 18S rRNA gene primers (forward primer: 5 -CTCGGTTCTATTTTGTCGGTTT-3 ; reverse primer: 5 GGCAAATGCTTT-CGCAGTAGT-3), bracketing a sequence of about 150 base pairs, were designed according to the conservative region of the fiddler crab, *Uca princeps* (GenBank accession no. 177767). NAG mRNA primers (forward primer: 5 -GGCATGGCAGCTGCTACA-3 ; reverse primer: 5 -GCGAGCGCTCCAAATTTGT-3) were designed based on the DNA sequencing result (Meng and Zou, 2009a). qRT-PCR was performed according to the protocols described in the QuantiTech SYBR Green PCR kit (Qiagen, Valencia, CA) on the Applied BioSystems StepOne Real-Time PCR System. 18S rRNA gene was used as endogenous control to indicate the NAG induction level in epidermal tissues. Amplification started with 50 °C for 30 min, first denaturing at 95 °C for 15 min, and followed by 40 cycles of amplification (94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s). The sample was increased to 95 °C for 15 s followed by a reduction to 60 °C for 1 min. The melting curve was increased from 60 °C to 95 °C at a rate of 0.1 °C/s. The sample remained at 95 °C for 15 s then was reduced to 40 °C for 15 s. The melting curve ensured that the correct DNA fragments were amplified. The PCR products of 18S rRNA and NAG mRNA have a melting temperature of 80.5 °C and 82.5 °C, respectively.

For the dose–response experiment, 5 intermolt crabs were randomly selected and injected with 0 (control), 0.2, 1, 5, and 20 ng/g wet weight MF, respectively. This experiment was repeated 7 times involving a total of 35 crabs. MF was first dissolved in ethanol, and then one volume of this MF solution in ethanol was mixed with 9 volumes of crab saline to get the injection solution. Control crabs were injected with an appropriate amount of crab saline with 10% v/v ethanol. One and half hours after injection, crabs were sacrificed and epidermal tissues were taken from beneath the carapace. The timing of sample collection was based on the previous finding that NAG gene transcription in the epidermis of *U. pugilator* is responsive to the molting hormone 1.5 h after hormonal treatment (Meng and Zou, 2009a). The total RNA of epidermal tissues from each crab was extracted and NAG mRNA quantified using the method described above. The relative quantity values of NAG mRNA were calculated using $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

To investigate whether the exogenous MF affects NAG activity, three groups of 25 intermolt crabs each were injected with 0 (control), 20, and 2000 ng/g wet weight MF, respectively, on day 0. On day 2, crabs were injected again with the same dosage of MF. After an additional 2 days, all survivors were sacrificed and epidermal tissue from beneath the carapace was extracted and homogenized on ice in 0.15 M pH 5.5 citrate-phosphate buffer containing proteinase inhibitor cocktail (Sigma, St. Louis, MO). After centrifugation at 10,000 *g* for 3 min, 20 μL of supernatant was incubated with 100 μL of 2 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO), a specific substrate for NAG, at 25 °C for 20 min. The reaction was stopped by the addition of 0.9 mL 0.5 M NaOH. The liberated nitrophenol was quantified at 405 nm with the Beckman DU730 Life Science UV/VIS Spectrophotometer. Protein concentrations in the supernatant were determined using the Bradford method. Enzymatic activity was expressed as μmol nitrophenol liberated $(\mu g \text{ protein})^{-1}$ $(20 \text{ min})^{-1}$ (Zou, 2009).

One-way analysis of variance (ANOVA) was used to determine the impacts of hormonal treatment on epidermal NAG mRNA and enzymatic activity. A probability value of less than 0.05 was deemed significant.

3. Results and discussion

No statistically significant difference was observed in NAG mRNA from the epidermis of control crabs and those injected with 0.2, 1, 5, or 20 ng/g MF ($p = 0.948$, Fig. 1), suggesting that the exogenous MF does