

The exogenous methyl farnesoate does not impact epidermal ecdysteroid signaling *in vivo* in the fiddler crab, *Uca pugilator*

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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 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 fiddler crabs 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 *Uca pugilator*. These results clearly show that MF is not capable of affecting epidermal ecdysteroid signaling in the fiddler crab, *Uca pugilator*. Our data are not supportive of the notion that MF signals through the RXR in Crustacea.

Key words: *N*-acetyl- β -glucosaminidase, ecdysteroid signaling, methyl farnesoate, RXR, *Uca pugilator*.

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 (Laufer *et al.* 1987; Landau *et al.* 1989). 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 & 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 & 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 *Daphnia 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 *Uca 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 human hepatocellular carcinoma cells (HepG2) transfected with daphnid RXR construct (Wang & 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 (Durica & Hopkins 1996; Chung *et al.* 1998). 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 and involved in degradation of exoskeletal chitin, has been unequivocally recognized as a product of the genes controlled by ecdysteroids in *Uca pugilator* (Zou & Fingerman 1999a,b; Meng & Zou 2009a,b).

The expression of NAG gene in the epidermis of *Uca 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. 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.

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 & Fingerman 1999a), only intermolt crabs (Vigh & Fingerman 1985) were used in the experiment.

Quantitative real-time PCR (qRT-PCR) was used to quantify epidermal NAG mRNA from *Uca 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 primers (Forward Primer: 5'-CTCGGTTCTATTTTGTCGGTTT-3'; Reverse primer: 5'GGCAAATGCTTTCGCAGTAGT-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 & 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 sec, 55°C for 30 sec, and 72°C for 30 sec). The sample was increased to 95°C for 15 seconds 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/sec. The sample remained at 95°C for 15 sec then was reduced to 40°C for 15 sec. 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 *Uca pugilator* is responsive to the molting hormone 1.5 hr after hormonal treatment (Meng & 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 & 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 10,000g for 3 min, 20 μ L of supernatant was incubated with 100 μ L 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 $(\mu\text{g protein})^{-1}(20 \text{ min})^{-1}$ (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.

Results

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 not affect epidermal NAG mRNA. At the enzymatic level, no significant difference was seen between NAG activities in the epidermis of control crabs and those receiving two injections of 20 or 2000 ng/g MF either ($p = 0.430$, Fig. 2).

Discussion

In the present study we show that the exogenous MF does not impact epidermal ecdysteroid signaling as measured by NAG mRNA in the fiddler crab. The validity of our finding depends, to a certain degree, on whether the MF doses used for injection are physiologically relevant. To our knowledge, there is still no data on MF titers in the hemolymph of *Uca pugilator*. The selection of MF injection doses was based on the range of MF

concentrations, <0.4 – 140 ng/ml, in the hemolymph of various decapod crustaceans (Nagaraju 2007). Since the blood volume of various decapods varies between 10 and 50% of the wet weight and the density of the blood in various decapods ranges from 1.025 to 1.052 (Maynard 1960), a mean blood volume/wet weight ratio of 30% and a blood density of 1.0 can be used to estimate the total blood volume of *Uca pugilator*. For instance, a crab of 2.0 g wet weight is estimated to have a blood volume of 0.6 mL. An injection dose at 1 ng/g wet weight would give rise to an MF concentration of about 3.3 ng/mL in the hemolymph. The injection doses of 0.2, 1.0, 5.0 and 20 ng/g wet weight for qRT-PCR experiments would give rise to estimated MF hemolymph concentrations of 0.7, 3.3, 17 and 670 ng/ml, respectively, well overlapping the reported MF range for decapods. The greater MF dose, 2000 ng/g wet weight, used for NAG activity induction experiment, which would result in an estimated MF concentration far exceeding the physiological range, was chosen to examine whether treatment with an excessive amount of MF would elicit any pharmacological effects on epidermal ecdysteroid signaling. The fact that none of the MF injection doses impacted NAG mRNA in the epidermis or affected epidermal NAG activity strongly indicates that MF has no bearing on epidermal ecdysteroid signaling as measured by NAG mRNA in *Uca pugilator*.

Although it is well known that MF functions as a hormone in Crustacea, its receptor remains equivocal. As mentioned above, because of the high affinity of MF for crustacean RXR and effects of a terpenoid mimic on the dynamics of daphnid RXR gene expression, Crustacean RXR has been proposed to be a candidate receptor for MF (Wang *et al.* 2007). In vertebrates, RXR, also known as the 9-*cis* retinoic acid receptor, can form homodimer RXR/RXR as well as heterodimers with dimerizing partners, such as retinoic acid receptors (RARs), thyroid hormone

receptors (TRs) and peroxisome proliferator-activated receptors (PPARs) (Mangelsdorf *et al.* 1995; Chambon 1996; Laflamme *et al.* 2002). The formation of RXR homodimer can be triggered by 9-*cis* retinoic acid (Zhang *et al.* 1992), and this homodimer can bind to the promoter and induce gene activation *in vivo* (Ijpenberg *et al.* 2004). These results suggest that the RXR/RXR dimer can mediate the signaling of 9-*cis* retinoic acid in vertebrates. The question here is: does crustacean RXR mediate the signaling of MF? The reporter gene assays performed with HepG2 cells transfected with the daphnid RXR have shown that MF, but not 9-*cis* retinoic acid, is inactive in transactivating the reporter gene, suggesting crustacean RXR alone cannot mediate MF signaling (Wang & LeBlanc 2009). Given that the presence of MF is known to be capable of altering the affinity of the EcR/RXR for the molting hormone (Hopkins *et al.* 2008), the next question becomes: can MF signal through the heterodimer EcR/RXR in Crustacea? The *in vitro* results seem to suggest that MF may signal through the EcR/RXR heterodimer. Wang & LeBlanc (2009) found that MF at 10 μM or 2500 ng/mL, but not at 1 μM , can enhance gene transactivation induced by 20-hydroxyecdysone in HepG2 cells transfected with both daphnid EcR and daphnid RXR. However, our *in vivo* data clearly show that MF does not impact epidermal ecdysteroid signaling. The discrepancy between our results and those of Wang & Leblanc (2009) may lie in the fact the reporter gene assays were performed with human cancer cells, whose molecular make-up may be different from that of crustacean cells. Additionally, two RXR isomers, UpRXR (-33) and UpRXR (+33), have been discovered in *Uca pugilator* (Wu *et al.* 2004), and the presence of MF can enhance the affinity of UpEcR/UpRXR for ponasterone A but reduce the affinity of UpEcR/UpRXR (+33) for such an ecdysteroid (Hopkins *et al.* 2008). It is likely that the cellular effects mediated by these two heterodimers would cancel out each other when MF is present, which could account for the absence of a significant effect of MF on

epidermal ecdysteroid signaling. Even if MF were capable of affecting ecdysteroid signaling through its interaction with crustacean RXR in the EcR/RXR heterodimer complex, the RXR should still not be regarded as the cognate receptor for MF, and MF is at most a modifier of molting hormone signaling. Therefore, the results of the present study, along with the finding that MF cannot activate RXR-mediated gene transcription (Wang & LeBlanc 2009), are not supportive of the notion that crustacean RXR is the receptor for MF.

Acknowledgements

This investigation was supported by the National Science Foundation EPSCoR Pfund grant NSF(2010)-PFUND-208 and the Louisiana sea grant undergraduate research opportunities program (UROP).

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Figure Captions

Figure 1. Effects of the exogenous methyl farnesoate on the mRNA of N-acetyl- β -glucosaminidase (NAG) in the epidermis of *Uca pugilator*. NAG mRNA is expressed in relative quantity. Sample size is shown in parenthesis. Error bars represent standard deviation.

Figure 2. Effects of MF on activity of N-acetyl- β -glucosaminidase (NAG) in the epidermis of *Uca pugilator*. Enzymatic activity was expressed as μmol nitrophenol liberated $(\mu\text{g protein})^{-1}(20 \text{ min})^{-1}$. Sample size is shown in parenthesis. Error bars represent standard deviation.

Figure 1

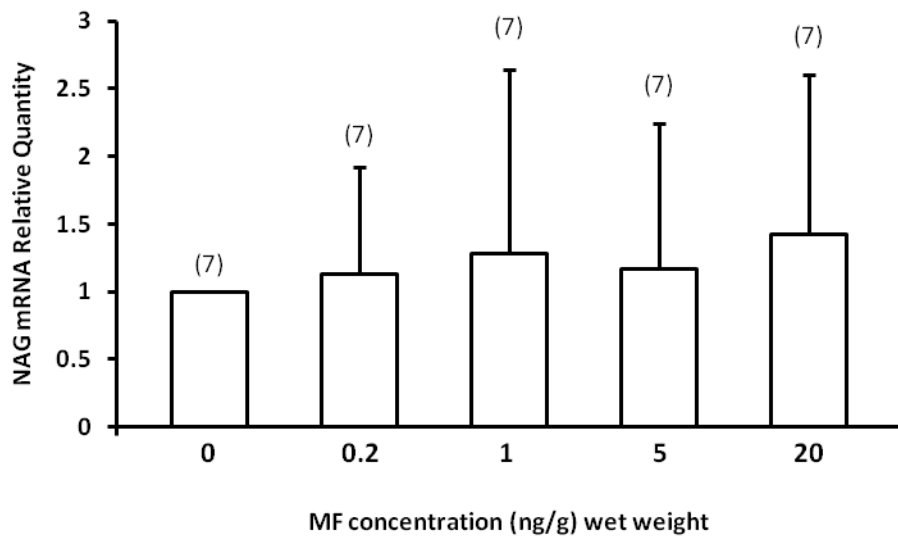


Figure 2

