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Negative regulation of juvenile hormone analog for ecdysteroidogenic enzymes

Mari H. Ogihara *, Juri Hikiba, Masatoshi Iga, Hiroshi Kataoka *

Graduate School of Frontier Sciences, The University of Tokyo, Kasiwano-ha 5-1-5, Kashiwa, Chiba 277-8562, Japan

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ABSTRACT

Disruption of the appropriate balance between juvenile hormone (JH) and ecdysteroids causes abnormal insect development. The application of a JH analog (JHA) during the early days of the final (fifth) instar induces dauer larvae with low ecdysteroid titers in insects, but the mechanism that underlies the action of JHA remains unclear. In this study, we clarified the negative effects of JHA on ecdysteroidogenic enzymes. JHA application to *Bombyx mori* larvae during the early stage of the fifth instar suppressed the expression of four enzymes, i.e., *neverland* (*nvd*), *spook*, *phantom*, and *disembodied* but not *non-molting glossy* and *shadow*. Furthermore, JHA application reduced the amount of 7-dehydrocholesterol, a metabolite produced by Nvd, in both the prothoracic glands and hemolymph, indicating JHA can disrupt ecdysteroidogenic pathway from the first step. Neck ligation resulted in increased *nvd* expression, whereas JHA application reversed this increase. These results suggest that the endogenous JH represses ecdysteroidogenesis during the early days in final instar larvae. Neck ligation and JHA application had no substantial effects on the expression of a transcription factor, *ftz-f1*, or a prothoracicotropic hormone receptor, *torso*; therefore, the inhibitory regulation of JHA may not involve these factors. Further analysis is required to clarify the regulation of JHA in ecdysteroidogenesis, but this study showed that JHA, and probably endogenous JH, can suppress the transcription of four of six ecdysteroidogenic enzymes. This regulation may be essential for maintaining the appropriate balance between JH and ecdysone during insect development.

1. Introduction

Insect development is regulated by two major hormones, juvenile hormone (JH), which maintains the larval status by preventing metamorphosis, and ecdysteroids, which trigger molting and metamorphosis. JH is synthesized in the corpora allata (CA), whereas ecdysteroids are synthesized in the prothoracic glands (PGs). These hormones are secreted into the hemolymph, and the titers of these hormones fluctuate in a stage-specific manner. The balance between these hormones in the hemolymph determines the developmental commitment from larva to pupa. Thus, the disruption of the appropriate balance between these hormones induces abnormal development. The absence of JH in the mutant CYP15C1, an essential cytochrome P450 monooxygenase (CYP) for JH synthesis, induces precocious metamorphosis (Daimon et al., 2012), whereas the absence of ecdysteroids because of ecdysteroidogenic enzyme gene mutations or gene silencing causes developmental arrest and insect death (Gilbert, 2004; Iga and Kataoka, 2012). By contrast, the exogenous application of these hormones causes different effects depending on when these hormones are applied (Sakurai and Imokawa, 1988; Sakurai et al., 1989; Dedos et al., 2002; Kamimura and Kiuchi, 2002). One of the most noticeable phenotypes caused by an application of a JH analog (JHA) is the dauer larva, which exhibits developmental arrest without undergoing molting or metamorphosis. In *Bombyx mori*, the application of JHA to larvae in the early stage of the final (fifth) instar induces the dauer larvae phenotype (Sakurai and Imokawa, 1988; Dedos and Fugo, 1999). This phenotype is similar to that observed in insects with ecdysteroidogenic enzyme deficiencies (Gilbert, 2004; Iga and Kataoka, 2012). Indeed, the ecdysteroid titers and secretory activities of PGs in dauer larvae are

Abbreviations: 2dE, 2-deoxyecdysone; 7dC, 7-dehydrocholesterol; 20E, 20-hydroxyecdysone; Br–CC–CA, brain–corpora cardiaca–corpora allata; C, cholesterol; CA, corpora allata; CYP, cytochrome P450 monooxygenase; Dib, disembodied; JH, juvenile hormone; JHA, juvenile hormone analog; KD, ketodiol; KT, ketotriol; NL, non-ligated; Nm-g, non-molting glossy; Nvd, neverland; PG, prothoracic gland; Phm, phantom; PTTH, prothoracicotropic hormone; Sad, shadow; Shd, shade; Spo, spook.

* Corresponding authors. Tel.: +81 04 7136 3627; fax: +81 04 7136 3626 (M.H. Ogihara). Tel.: +81 04 7136 3622; fax: +81 04 7136 3623 (H. Kataoka).

E-mail addresses: m-h-ogihara@ib.k.u-tokyo.ac.jp (M.H. Ogihara), kataoka@k.u-tokyo.ac.jp (H. Kataoka).

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much lower than those in normal larvae (Sakurai and Imokawa, 1988; Dedos and Fugo, 1996). JHA application is expected to regulate ecdysteroidogenesis negatively, but the mechanism is unclear.

Ecdysteroid synthesis occurs in PGs via sequential hydroxylation by ecdysteroidogenic enzymes (Iga and Kataoka, 2012). Ecdysteroidogenesis is initiated with cholesterol (C) and a Rieske oxygenase, Neverland (Nvd) (Yoshiyama et al., 2006; Yoshiyama-Yanagawa et al., 2011). The enzyme converts C into 7-dehydrocholesterol (7dC). Several of the reactions between 7dC and ketodiol (KD) have not yet been determined, but at least two enzymes are required, i.e., a short-chain dehydrogenase/reductase Non-molting glossy (Nm-g) and CYP307A1 (Spook, Spo) (Namiki et al., 2005; Ono et al., 2006; Sztal et al., 2007; Niwa et al., 2010). The reactions that utilize CYP306A1 (Phantom, Phm), CYP302A1 (Disembodied, Dib), and CYP315A1 (Shadow, Sad) convert KD into ketotriol (KT), 2-deoxyecdysone (2dE), and ecdysone, respectively. Ecdysone is secreted from PGs into the hemolymph, and CYP314A1 (Shade, Shd) converts it into 20-hydroxyecdysone (20E), an active form of ecdysteroid, in the peripheral tissues (Petryk et al., 2003; Rewitz et al., 2006; Maeda et al., 2008). The organized expression of these enzymes induces fluctuations in the ecdysteroid titer, whereas suppression of any of these enzymes reduces the ecdysteroid titer. The dauer larvae possess fewer ecdysteroids, so JHA may inhibit the regulation of ecdysteroidogenic enzymes. Therefore, we investigated the negative effects of JHA on these enzymes. In this study, we clarified the targets of JHA, and thus, we discuss the possibility that endogenous JH is the innate negative regulator of ecdysteroidogenesis.

2. Materials and methods

2.1. Chemicals

Fenoxycarb (JHA) was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). Fenoxycarb was dissolved in acetone (Wako, Osaka, Japan) and was stored at −20 °C until further use. C, 7dC, ecdysone, and 20E were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Insects and JHA application to larvae

B. mori (Kinshu × Showa, F1 hybrid) larvae were used in this study. *B. mori* larvae were reared on an artificial diet called silkmate (Nihon-Nosan, Yokohama, Kanagawa, Japan) at 25 °C under a 16/8 h light/dark cycle. One microgram of fenoxycarb in 5 μl acetone (JHA application) or 5 μl of acetone alone (control) was applied topically to the dorsal surface of day 2 fifth instar larvae or neck-ligated larvae. The neck-ligated larvae were prepared by withholding diet from fourth instar larvae after head capsule slippage and by segregating the newly molted fifth instar larvae prior to feeding. The larvae were then ligated at the neck and decapitated. The JHA- or acetone-treated larvae were reared separately in plastic cases at 25 °C under a 16/8 h light/dark cycle.

2.3. qRT-PCR

PGs were dissected from larvae after JHA or acetone application, and the total RNA was extracted from PGs using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was purified with RQ DNase I (Promega, Madison, WI, USA), and cDNA was synthesized using a PrimeScript RT Reagent Kit (Perfect Real Time). SYBR Premix Ex Taq II (Perfect real-time) (TaKaRa Bio, Otsu, Shiga, Japan) was used in the experiments where JHA or acetone was applied to day 2 fifth instar larvae, and SYBR Premix Ex Taq II (T li RNase plus, TaKaRa Bio) was used in the other experiments. The primers used in this study are listed in Table 1. *Rpl3* was used as an internal control, and relative expression levels were determined. The expression levels of the respective genes were normalized against those in the control larvae at 1 day after acetone application (Fig. 1). To evaluate the effects of JHA application on neck-ligated larvae, the expression levels of the target genes were normalized against those of non-ligated 0 day fifth instar larvae (NL, Fig. 3). The PCR conditions used to amplify *nvd*, *nm-g*, *spo*, *phm*, and *rpl3* comprised 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and at 68 °C for 30 s. The PCR conditions for *dib* and *sad* comprised 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 30 s. A Thermal Cycler Dice Real Time System (TaKaRa Bio) was used to perform qRT-PCR. The statistical significance of the expression levels was evaluated by a Welch *t*-test.

2.4. Purification and quantification of ecdysteroids and their intermediates by LC–MS/MS

PGs were dissected daily from larvae that received JHA or acetone applications on day 2 of the fifth instar larvae. PGs were sonicated in buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 2 mM EGTA), and the sterols in the buffer were extracted with two volumes of 1-butanol, as described previously (Hikiba et al., 2013). The sterols obtained were analyzed by LC–MS/MS, as described previously (Hikiba et al., 2013). Hemolymph (100 μl) was collected from day 10 fifth instar larvae that received JHA or acetone applications on day 2 of the fifth instar larvae. The sterols were extracted from the hemolymph samples using nine volumes of methanol (Wako). The samples were vortexed and centrifuged at 17,800×*g* for 10 min at 4 °C. The supernatant was collected and filtered through a Millex-LG Syringe Driven Filter Unit (φ 0.20 μm) (Millipore, Carslbad, CA, USA). The extracted sterols were analyzed by LC–MS/MS, as described previously (Iga et al., 2014). The statistical significance of the level of 7dC was evaluated by a Welch *t*-test.

3. Results

3.1. Suppression of genes encoding ecdysteroidogenic enzymes by JHA application

We determined the expression levels of *nvd*, *nm-g*, *spo*, *phm*, *dib*, and *sad* after acetone or JHA application by qRT-PCR (Fig. 1). The control larvae initiated wandering approximately 7 days after molting, whereas the larvae that received an application of JHA

Table 1
Primers used in this study.

Genes		Sequences (5′ → 3′)	References
nvd	F	TCTTCGAACACGGCGTGCCC	Iga et al. (2014)
	R	ACAAAATCGCGGAGAACGCAAAC	Iga et al. (2014)
nm-g	F	TGTGATAGTGGACTCGGTTGGGC	Iga et al. (2014)
	R	GGCTTTCGCTGCTTCGGTTTC	Iga et al. (2014)
spo	F	CTACACGAAGACCCGACCAT	Iga et al. (2014)
	R	GCGTCGATTTCCTGACGTAT	Iga et al. (2014)
phm	F	GACCCAACGGCACTGTATATGAGAG	Niwa et al. (2004)
	R	GCCCACTGCAATGGGATCAC	Niwa et al. (2004)
dib	F	ACAAGACCACCGCTATCCAG	
	R	GTTGGTCGCAAGAAGTCCTC	
sad	F	TATCAGCGACCCAACTCACA	
	R	CCAATCCTCTCCGTCCATAA	
ftz	F	CAGCGGCTACCACTATGGAT	
	R	CTCGAACCGCTTCAAGTTTC	
torso	F	TCAGGATTGGATGGAAGGTC	
	R	AACGAATTTTCCGTTGATCG	
rpl3	F	TGGCACACAAAGAAGCTACCC	Iga et al. (2014)
	R	TGACCAGCACGAGCTACAGTG	Iga et al. (2014)