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Roles of mechanistic target of rapamycin and transforming growth factor-β signaling in the molting gland (Y-organ) of the blackback land crab, *Gecarcinus lateralis*

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ABSTRACT

Molting in decapod crustaceans is controlled by molt-inhibiting hormone (MIH), an eyestalk neuropeptide that suppresses production of ecdysteroids by a pair of molting glands (Y-organs or YOs). Eyestalk ablation (ESA) activates the YOs, which hypertrophy and increase ecdysteroid secretion. At mid premolt, which occurs 7–14 days post-ESA, the YO transitions to the committed state; hemolymph ecdysteroid titers increase further and the animal reaches ecdysis ~3 weeks post-ESA. Two conserved signaling pathways, mechanistic target of rapamycin (mTOR) and transforming growth factor-β (TGF-β), are expressed in the *Gecarcinus lateralis* YO. Rapamycin, an mTOR antagonist, inhibits YO ecdysteroidogenesis *in vitro*. In this study, rapamycin lowered hemolymph ecdysteroid titer in ESA *G. lateralis in vivo*; levels were significantly lower than in control animals at all intervals (1–14 days post-ESA). Injection of SB431542, an activin TGF-β receptor antagonist, lowered hemolymph ecdysteroid titers 7 and 14 days post-ESA, but had no effect on ecdysteroid titers at 1 and 3 days post-ESA. mRNA levels of mTOR signaling genes *Gl-mTOR*, *Gl-Akt*, and *Gl-S6k* were increased by 3 days post-ESA; the increases in *Gl-mTOR* and *Gl-Akt* mRNA levels were blocked by SB431542. *Gl-elongation factor 2* and *Gl-Rheb* mRNA levels were not affected by ESA, but SB431542 lowered mRNA levels at Days 3 and 7 post-ESA. The mRNA level of an activin TGF-β peptide, *Gl-myostatin-like factor* (*Mstn*), increased 5.5-fold from 0 to 3 days post-ESA, followed by a 50-fold decrease from 3 to 7 days post-ESA. These data suggest that (1) YO activation involves an up regulation of the mTOR signaling pathway; (2) mTOR is required for YO commitment; and (3) a Mstn-like factor mediates the transition of the YO from the activated to the committed state.

1. Introduction

Control of molting in crustaceans involves a complex interaction between the eyestalk neurosecretory center, which produces inhibitory neuropeptides, such as molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH), and a pair of molting glands (Y-organs or YOs) in the anterior cephalothorax (Chang and Mykles 2011; Hopkins 2012; Webster 2015). MIH maintains the YO in the basal state during intermolt (stage C_4) through a cyclic nucleotide second messenger pathway (Chang and Mykles 2011; Covi et al. 2009, 2012). A reduction in MIH activates the YO and triggers the transition from intermolt to premolt (stage D_0). Molting is induced by eyestalk ablation (ESA) or multiple leg autotomy in many decapod species, including *Gecarcinus lateralis* (Chang and Mykles 2011; Mykles 2001). The activated YO hypertrophies to increase molting hormone (ecdysteroids) synthetic capacity (Chang and Mykles 2011; Mykles 2011). The YO remains sensitive to MIH, CHH, and other factors, so that premolt processes can be temporally suspended by stress or injury (e.g., limb bud autotomy or LBA) (Chang and Mykles 2011; Mykles 2001; Nakatsuji et al. 2009; Yu et al. 2002). By mid premolt (stage D_{1-2}), the YO transitions to the committed state, in which ecdysteroid production increases further and the YO becomes insensitive to MIH, CHH, and LBA (Chang and Mykles 2011; Mykles 2001; Nakatsuji et al. 2009). Increased phosphodiesterase (PDE) activity contributes to the reduced response to MIH by keeping intracellular cyclic nucleotides low (Chang and Mykles 2011; Nakatsuji et al. 2009). By the end of premolt (stage D_{3-4}), high ecdysteroids initiate the transition from the committed state to the repressed state; hemolymph ecdysteroid titers drop precipitously and the animal molts (Chang and Mykles 2011; Mykles 2011).

The signaling pathways that drive the changes in the YO during the premolt period are poorly understood. In insects, the insulin/mechanistic target of rapamycin (mTOR) signal transduction pathway regulates

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prothoracic gland (PG) growth and ecdysteroidogenic capacity (see (Danielsen et al. 2013; Nijhout et al. 2014; Rewitz et al. 2013; Yamanaka et al. 2013) for reviews). mTOR is a protein kinase highly conserved among the Metazoa that functions as a sensor for cellular growth regulation by nutrients, cellular energy status, oxygen level, and growth factors (Albert and Hall 2015; Cetrullo et al. 2015; Laplante and Sabatini 2013). Prothoracicotropic hormone (PTTH) and insulin-like peptides (ILPs) activate mTOR, which phosphorylates p70 S6 kinase (S6K) and eIF4E-binding protein to increase global translation of mRNA into protein (Smith et al. 2014; Teleman 2010; Yamanaka et al. 2013). FK506-binding protein 12 complexes with rapamycin to inhibit mTOR (Hausch et al. 2013). Binding of ILP to a membrane receptor activates a signal transduction cascade involving PI3K, PDK1, and Akt protein kinases (Teleman 2010). mTORC1 is activated by Rheb-GTP and is inactivated when Rheb-GTPase activating protein (Rheb-GAP or tuberous sclerosis complex 1/2) promotes the hydrolysis of GTP to GDP by Rheb (Huang and Manning 2008). Phosphorylation by Akt inactivates Rheb-GAP; the higher Rheb-GTP levels keep mTOR in the active state (Teleman 2010). Over-expressing Rheb-GAP inhibits PG growth, while over-expressing PI3K, an upstream activator of Akt, stimulates PG growth (Colombani et al. 2005; Layalle et al. 2008; Mirth et al. 2005). In addition, inhibition of PI3K and mTOR blocks the PTTH-dependent increase in ecdysteroid secretion in the PG (Gu et al. 2012; Gu et al. 2011). In *G. lateralis*, rapamycin inhibits YO ecdysteroid secretion *in vitro* and the expression of *Gl-mTOR* and *Gl-Akt* is increased in animals induced to molt by multiple leg autotomy, suggesting that mTOR signaling is involved in YO activation (Abuhagr et al. 2014b).

The transforming growth factor-β (TGF-β) superfamily is mediated by Smad transcription factors that regulate genes through transcriptional activation or repression (Heldin and Moustakas 2012; Macias et al. 2015; Xu et al. 2012). TGFβ/Smad signaling controls PTTH-stimulated ecdysteroidogenesis in the insect PG (Rewitz et al. 2013; Yamanaka et al. 2013). Disruption of activin (Actb) signaling in *Drosophila* blocks the metamorphic molt by preventing the ecdysteroid peak by PTTH (Gibbens et al. 2011). Activin is required for the PG to respond to PTTH; animals do not molt until they have achieved a critical weight (Rewitz et al. 2013). An activin-like peptide may have a similar function in crustaceans, as the committed YO shows a sustained constitutive increase in ecdysteroid synthesis and reduced sensitivity to MIH (Chang and Mykles 2011; Nakatsuji et al. 2009).

The components of the mTOR and TGF-β signaling pathways are well represented in the *G. lateralis* YO transcriptome (Das et al. 2016). The purpose of this study is to investigate the roles of mTOR and TGF-β signaling in regulating YO ecdysteroidogenesis. ESA was used to induce molting in *G. lateralis*. The effects of rapamycin, an mTOR inhibitor, on hemolymph ecdysteroid titer and of SB431542, an activin receptor antagonist, on ecdysteroid titer and gene expression *in vivo* were determined. Hemolymph ecdysteroid titer was quantified by competitive ELISA. mRNA levels of *Gl-elongation factor 2* (*Gl-EF2*), *Gl-myostatin-like factor* (*Gl-Mstn*), *Gl-mTOR*, *Gl-Rheb*, *Gl-Akt*, and *Gl-S6k* were quantified by quantitative polymerase chain reaction (qPCR). The results suggest that mTOR and activin signaling control YO ecdysteroidogenesis during premolt.

2. Materials and methods

2.1. Animals and experimental treatments

Adult blackback land crabs, *G. lateralis*, were collected in the Dominican Republic, shipped via commercial air cargo to Colorado, USA, and maintained as described previously (Covi et al. 2010). Molting was induced by eyestalk ablation (Covi et al. 2010; MacLea et al. 2012).

The effects of SB431542 and rapamycin were determined *in vivo*. At Day 0, intermolt *G. lateralis* was ES-ablated and received a single injection of 10 mM SB431542 (Selleck Chemicals, Houston, TX, USA) or 10 mM rapamycin (Selleck Chemicals) in dimethyl sulfoxide (DMSO; ~10 μM estimated final hemolymph concentration) or dimethyl sulfoxide (DMSO; ~0.1% estimated final hemolymph concentration). Intact intermolt animals also received SB431542 or DMSO. The injection volume was based on an estimated hemolymph volume of 30% of the wet weight. It was calculated using the equation: g wet weight × 0.3 μl 10 mM SB431542, 10 mM rapamycin, or DMSO. YOs were harvested at 0, 1, 3, 5 (*Gl-Mstn* only), 7, and 14 days post-injection, frozen in liquid nitrogen, and stored at −80 °C. Hemolymph samples (100 μl) were taken at the time of tissue harvesting, mixed with 300 μl methanol, and ecdysteroid was quantified by ELISA (Abuhagr et al. 2014a).

2.2. Expression of mTOR signaling genes in Y-organ

Total RNA was isolated from YOs using TRIzol reagent (Life Technologies, Carlsbad, CA) as described previously (Covi et al. 2010). First-strand cDNA was synthesized using 2 μg total RNA in a 20 μl total reaction with SuperScript III reverse transcriptase (Life Technologies) and oligo-dT(20)VN primer (50 μmol/l; IDT, Coralville, IA) as described (Covi et al. 2010).

A LightCycler 480 thermal cycler (Roche Applied Science, Indianapolis, IN) was used to quantify levels of *Gl-EF2* (GenBank AY552550), *Gl-Mstn* (EU432218), *Gl-mTOR* (HM989973), *Gl-Rheb* (HM989970), *Gl-Akt* (HM989974), and *Gl-S6k* (HM989975) mRNAs (Covi et al. 2008, 2010; MacLea et al. 2012). Reactions consisted of 1 μl first strand cDNA or standard, 5 μl 2× SYBR Green I Master mix (Roche Applied Science), 0.5 μl each of 10 mM forward and reverse primers synthesized by Integrated DNA Technologies (IDT; Coralville, Iowa; Table 1), and 3 μl nuclease-free water. PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 20 s, and extensions at 72 °C for 20 s, followed by melting curve analysis of the PCR product. Transcript concentrations were determined with the LightCycler 480 software (Roche, version 1.5) using a series of dsDNA gene standards produced by serial dilutions of PCR product for each gene (10 ag/μl to 10 ng/μl). The absolute amounts of transcript in copy numbers per μg of total RNA in the cDNA synthesis reaction were calculated based on the standard curve and the calculated molecular weight of dsDNA products.

2.3. Statistical analyses and software

Statistical analysis was performed using JMP 12.1.0 (SAS Institute, Cary, NC). Means were compared using one-way analysis of variance (ANOVA), both within and between treatments. *Post-hoc* Tukey tests were additionally used to compare means over time within a treatment group. Data are presented as mean ± 1 S.E. and the level of significance for all of the data analyses was set at $\alpha = 0.05$. All qPCR data was log

Table 1
Oligonucleotide primers used for gene expression analysis (qPCR).

Primer	Sequence (5′-3′)	Product size (bp)
Gl-EF2 F1	TTCTATGCCTTTGGCCGTGTCTTCTC	227
Gl-EF2 R1	ATGGTGCCCGTCTTAACCA	
Gl-Mstn F1	GCTGTCGCCGATGAAGATGT	118
Gl-Mstn R1	GGCTGGGGACCTCAATCCCGT	
Gl-mTOR F2	AGAAGATCCTGCTGAACATCGAG	159
Gl-mTOR R2	AGGAGGGACTCTTGAACCACAG	
Gl-Rheb F1	TTTGTGGACAGCTATGATCCC	119
Gl-Rheb R1	AAGATGCTATACTCATCCTGACC	
Gl-Akt F2	AACTCAAGTACTCCAGCGATGATG	156
Gl-Akt R1	GGTTGCTACTCTTTTCACGACAGA	
Gl-s6k F2	GGACATGTGAAGCTCACAGACTTT	239
Gl-s6k R1	TTCCCCTTCAGGATCTTCTCTATG	

Abbreviations: Gl, *G. lateralis*; F, forward; R, reverse; Akt, protein kinase B; EF2, elongation factor 2; Mstn, myostatin-like factor; mTOR, mechanistic target of rapamycin; Rheb, Ras homolog expressed in brain; and s6k, p70 S6 kinase.