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Stimulation of orphan nuclear receptor *HR38* gene expression by PTTH in prothoracic glands of the silkworm, *Bombyx mori*

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ABSTRACT

A complex signaling network appears to be involved in prothoracicotropic hormone (PTTH)-stimulated ecdysteroidogenesis in insect prothoracic glands (PGs). Less is known about the genomic action of PTTH signaling. In the present study, we investigated the effect of PTTH on the expression of *Bombyx mori HR38*, an immediate early gene (IEG) identified in insect systems. Our results showed that treatment of *B. mori* PGs with PTTH *in vitro* resulted in a rapid increase in *HR38* expression. Injection of PTTH into day-5 last instar larvae also greatly increased *HR38* expression, verifying the *in vitro* effect. Cycloheximide did not affect induction of *HR38* expression, suggesting that protein synthesis is not required for PTTH's effect. A mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor (U0126), and a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002), partially inhibited PTTH-stimulated *HR38* expression, implying the involvement of both the ERK and PI3K signaling pathways. When PGs were treated with agents that directly elevate the intracellular Ca^{2+} concentration (either A23187 or thapsigargin), an increase in *HR38* expression was also detected, indicating that Ca^{2+} is involved in PTTH-stimulated *HR38* gene expression. A Western blot analysis showed that PTTH treatment increased the HR38 protein level, and protein levels showed a dramatic increase during the later stages of the last larval instar. Expression of *HR38* transcription in response to PTTH appeared to undergo development-specific changes. Treatment with ecdysone *in vitro* did not affect *HR38* expression. However, 20-hydroxyecdysone treatment decreased *HR38* expression. Taken together, these results demonstrate that *HR38* is a PTTH-stimulated IEG that is, at least in part, induced through Ca^{2+}/ERK and PI3K signaling. The present study proposes a potential cross talk mechanism between PTTH and ecdysone signaling to regulate insect development and lays a foundation for a better understanding of the mechanisms of PTTH's actions.

1. Introduction

Nuclear receptors (NRs) comprise a superfamily of structurally related transcriptional factors that play important roles in regulating eukaryotic cell growth, development, and homeostasis (Evans and Mangelsdorf, 2014). The family includes ligand-inducible NRs and a number of so-called orphan receptors that lack identified physiological ligands (Benoit et al., 2006; Evans and Mangelsdorf, 2014). In mammals, it was reported that the NR4A receptor family belongs to the orphan NR superfamily and consists of three highly homologous mammalian members known as Nur77 (also referred to as NR4A1 and NGFI-B), Nurr1 (NR4A2), and NOR-1 (NR4A3) (Close et al., 2013; Kurakula et al., 2014). All of the NR4A subfamilies are encoded by immediate early genes (IEGs) the expressions of which are rapidly induced in response to a variety of signals including mitogens and cellular stress (Maxwell and Muscat, 2006). Moreover, NR4A receptors are widely expressed in several tissues, including the testis, ovary, muscle, thymus, adrenal gland, and brain (Maxwell and Muscat, 2006; Pei et al., 2006). In line with pleiotropic physiological stimuli that induce NR4A expression, the NR4A family appears to play important roles in regulating cell metabolism and homeostasis. The role of NR4A in regulating mammalian steroidogenesis was also documented (Maxwell and Muscat, 2006). Nur77 and Nurr1 were shown to play key roles in regulating basal and hormone-induced gene expressions in steroidogenic cells, including testicular Leydig cells (Maxwell and Muscat, 2006).

In insects, both *Drosophila* HR38 (DHR38) and *Bombyx* HR38 belong to the NR4A subfamily of NRs and appear to disrupt ecdysone receptor (EcR)-Ultraspiracle (USP) heterodimers *in vitro* and in cell culture (Sutherland et al., 1995). Dimerization of HR38 with

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USP blocks ecdysone signaling *in vivo* during the arrest that precedes a blood meal in *Aedes aegypti* (Zhu et al., 2000). Evidence suggests that DHR38 participates with USP in an unusual ecdysteroid signaling pathway in *Drosophila* (Baker et al., 2003). DHR38 also plays pivotal roles in adult cuticle formation, carbohydrate metabolism, and glycogen storage (Kozlova et al., 1998, 2009; Ruaud et al., 2011). More recently, *HR38* was identified as a novel IEG that is transiently expressed in the male by sex pheromone in both *Drosophila* and *Bombyx* and thus *HR38* can be used as a neural activity marker in insect brains (Fujita et al., 2013).

Insects molting and metamorphosis are initiated by ecdysteroids synthesized and secreted by prothoracic glands (PGs) (Thummel, 2001; De Loof, 2011; Smith and Rybczynski, 2012; De Loof et al., 2013, 2015). A neuropeptide, known as the prothoracicotropic hormone (PTTH), produced by brain neurosecretory cells, activates ecdysteroidogenesis in PGs (Ishizaki and Suzuki, 1994; Marchal et al., 2010; Smith and Rybczynski, 2012; De Loof et al., 2013, 2015). PTTH appears to bind to receptor tyrosine kinase to initiate a signaling transduction network (Rewitz et al., 2009, 2013; Marchal et al., 2010; Smith and Rybczynski, 2012; De Loof et al., 2013, 2015). Previous studies indicated that PTTH-stimulated cAMP and Ca^{2+} are intracellular second messengers in both *Manduca sexta* (Smith et al., 1984, 1985; Fellner et al., 2005) and *Bombyx mori* (Gu et al., 1996, 1998). In *M. sexta*, p70S6 kinase (S6K) and ribosomal protein S6 are related to PTTH-stimulated ecdysteroidogenesis (Song and Gilbert, 1997). In addition, extracellular signal-regulated kinase (ERK) phosphorylation, phosphatidylinositol 3-kinase (PI3K)/adenosine 5′-monophosphate-acti vated protein kinase (AMPK)/target of rapamycin (TOR) signaling, and reactive oxygen species were found to be involved in PTTH's stimulation of ecdysteroidogenesis (Rybczynski et al., 2001; Lin and Gu, 2007; Gu et al., 2010, 2011, 2012, 2013; Hsieh et al., 2013, 2014).

Our recent study further characterized the nuclear localization of phosphorylated ERK stimulated by PTTH (Gu and Hsieh, 2015). We demonstrated that PTTH stimulates phosphorylation of histone H3 at ser10 in *B. mori* PGs both *in vitro* and *in vivo* (Gu and Hsieh, 2015). In mammalian cells, it was well documented that phosphorylation of histone H3 at ser10 in response to a mitogenic stimulus closely corresponds to transient expressions of activated IEGs, suggesting that this histone modification is linked to transcription activation (Prigent and Dimitrov, 2003; Sawicka and Seiser, 2014). Although PTTH appears to regulate expression of some ecdysteroidogenic genes (Niwa et al., 2005; Yamanaka et al., 2007; McBrayer et al., 2007), no IEG has been identified in PTTH-stimulated PGs.

In addition, transcriptional changes stimulated by the activation of EcR have been well characterized in *Drosophila* (Thummel, 2001; King-Jones and Thummel, 2005; Niwa and Niwa, 2014, 2016). It is hypothesized that some components of the EcR may be involved in the biosynthesis of ecdysone. EcRA, but not the two other EcR isoforms, is expressed in *Drosophila* PGs (Talbot et al., 1993). In *Manduca*, USP appears to modulate PTTH-stimulated ecdysteroidogenesis (Song and Gilbert, 1998). A null mutation in *E75A* results in a dramatic decrease in ecdysone levels, implying that E75A plays a dual role, acting both downstream of ecdysone signaling and upstream of ecdysone biosynthesis signaling (Bialecki et al., 2002; Li et al., 2015). Other NRs, such as βFTZ-F1 and Broad, appear to play similar roles in regulating both ecdysone's action and its biosynthesis (Parvy et al., 2005; Xiang et al., 2010). It was also reported that PTTH signaling mediates nucleocytoplasmic trafficking of the NR DHR4, which acts as a repressor of ecdysteroidogenesis (Ou et al., 2011). More recently, it was shown that EcR-dependent positive feedback operating downstream of PTTH signaling plays a critical role in generating the high-level pulse that triggers pupariation in response to PTTH (Moeller et al., 2013). Complex interaction among the NRs, EcR, E75, DHR3, and βFTZ-F1, which mediate ecdysone signaling (King-Jones and Thummel, 2005), appears to be involved in regulating ecdysteroidogenesis (Parvy et al., 2014). Although those studies clearly indicated the involvement of the NRs in regulating ecdysteroidogenesis, the link from PTTH signaling to the NRs is not very clear.

Considering that NR4A expression is induced by pleiotropic physiological stimuli and that NR4A plays an important role in regulating steroidogenesis in mammals, in the present study, we performed a detailed analysis of HR38 expression at both mRNA and protein levels in response to PTTH signaling in *B. mori* PGs. We demonstrate that *HR38* is an IEG downstream of PTTH signaling, and thus appears to be involved in PTTH-stimulated ecdysteroidogenesis.

2. Materials and methods

2.1. Experimental animals

Larvae of an F1 racial hybrid, Guofu × Nongfong, were reared on fresh mulberry leaves at 25 °C under a 12-h light: 12-h dark photoperiod. Newly-ecdysed 4th and last instar larvae were collected and used for each experiment.

2.2. Reagents and antibodies

Ecdysone, 20-hydroxyecdysone, A23187, and thapsigargin were supplied by Sigma-Aldrich (St. Louis, MO, USA). Grace's insect cell culture medium was obtained from Invitrogen (Carlsbad, CA, USA). A mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor (U0126), a PI3K inhibitor (LY294002), and a protein synthesis inhibitor (cycloheximide, CHX) were purchased from Calbiochem (San Diego, CA, USA). All other reagents used were of analytical grade. Recombinant *B. mori* PTTH (PTTH) was produced by infection of *Spodoptera frugiperda*-SF21 cells with the vWTPTTHM baculovirus as previously described (O'Reilly et al., 1995). The same PTTH as that previously reported (O'Reilly et al., 1995; Gu et al., 2010; Gu and Hsieh, 2015) was used in the present study. In the present study, extracellular fluid from cells infected with vWTPTTHM rather than pure PTTH was used as the PTTH source, and it was diluted 500 times with medium. Each incubation (50 μl) contained about 0.15 ng PTTH.

An antibody directed against *Bombyx* HR38 was kindly provided by Dr. Taketoshi Kiya (Fujita et al., 2013). An anti-α-tubulin antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). A horseradish peroxidase (HRP)-linked goat anti-rabbit second antibody was purchased from PerkinElmer Life Sciences (Boston, MA, USA).

2.3. In vitro incubation of PGs and in vivo injection of PTTH

PGs from day-5 last instar larvae or other stages were dissected in lepidopteran saline (Gu et al., 2010). Following dissection, the medium was replaced with fresh medium (with or without inhibitors), and a 30-min preincubation period was initiated. After preincubation, glands were rapidly transferred to fresh medium (with or without experimental materials, such as an inhibitor or PTTH) and then incubated with gentle shaking. After incubation, glands were flash-frozen at −70 °C for a subsequent sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. To study the *in vivo* effect of PTTH on *HR38* expression, day-5 last instar larvae were injected with 10 μl saline containing 0.3 μl of the original PTTH solution. Larvae injected with only 10 μl saline were used as the controls.