Insect Biochemistry and Molecular Biology 41 (2011) 197–202

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Involvement of PI3K/Akt signaling in PTTH-stimulated ecdysteroidogenesis by prothoracic glands of the silkworm, *Bombyx mori*

Shi-Hong Gu*, Shun-Chieh Young, Ju-Ling Lin, Pei-Ling Lin

Department of Zoology, National Museum of Natural Science, 1 Kuan-Chien Road, Taichung 404, Taiwan, ROC

ARTICLE INFO

Article history:
Received 31 May 2010
Received in revised form
11 December 2010
Accepted 19 December 2010

Keywords:
Bombyx mori
PTTH
Ecdysteroidogenesis
Prothoracic gland
PI3K
Akt
Signal transduction
Crosstalk

ABSTRACT

The prothoracicotropic hormone (PTTH) stimulates ecdysteroidogenesis by prothoracic gland in larval insects. Previous studies showed that Ca^{2+}, cAMP, extracellular signal-regulated kinase (ERK), and tyrosine kinase are involved in PTTH-stimulated ecdysteroidogenesis by the prothoracic glands of both *Bombyx mori* and *Manduca sexta*. In the present study, the involvement of phosphoinositide 3-kinase (PI3K)/Akt signaling in PTTH-stimulated ecdysteroidogenesis by *B. mori* prothoracic glands was further investigated. The results showed that PTTH-stimulated ecdysteroidogenesis was partially blocked by LY294002 and wortmannin, indicating that PI3K is involved in PTTH-stimulated ecdysteroidogenesis. Akt phosphorylation in the prothoracic glands appeared to be moderately stimulated by PTTH in vitro. PTTH-stimulated Akt phosphorylation was inhibited by LY294002. An in vivo PTTH injection into day 6 last instar larvae also increased Akt phosphorylation of the prothoracic glands. In addition, PTTH-stimulated ERK phosphorylation of the prothoracic glands was not inhibited by either LY294002 or wortmannin, indicating that PI3K is not involved in PTTH-stimulated ERK signaling. A23187 and thapsigargin, which stimulated *B. mori* prothoracic gland ERK phosphorylation and ecdysteroidogenesis, could not activate Akt phosphorylation. PTTH-stimulated ecdysteroidogenesis was not further activated by insulin, indicating the absence of an additive action of insulin and PTTH on the prothoracic glands. The present study, together with the previous demonstration that insulin stimulates *B. mori* ecdysteroidogenesis through PI3K/Akt signaling, suggests that crosstalk exists in *B. mori* prothoracic glands between insulin and PTTH signaling, which may play a critical role in precisely regulated ecdysteroidogenesis during development.

1. Introduction

Ecdysteroids, synthesized by the prothoracic glands of larval insects, are essential for driving the molecular and cellular events that lead to molting and metamorphosis (Gilbert et al., 2002; Rybczynski, 2005; Marchal et al., 2010). Ecdysteroidogenesis in prothoracic glands is activated by the prothoracicotropic hormone (PTTH), a neuropeptide produced by the brain (Gilbert et al., 2002; Rybczynski, 2005). Numerous studies have investigated PTTH signaling transduction. Results indicate that PTTH stimulation of ecdysteroid synthesis appears to be mediated by cAMP and Ca^{2+} as the intracellular second messengers in both *Manduca sexta* (Smith et al., 1984, 1985; Fellner et al., 2005) and *Bombyx mori* (Gu et al., 1996, 1997, 1998, 2000). In *M. sexta,* it was reported that p70S6 kinase and ribosomal protein S6 are related to PTTH-stimulated ecdysteroidogenesis (Song and Gilbert, 1994, 1995, 1997). In addition, the involvement of extracellular signal-regulated kinase (ERK) phosphorylation in PTTH stimulation of ecdysteroidogenesis was documented (Rybczynski et al., 2001; Lin and Gu, 2007). Further investigation showed that Torso, a receptor tyrosine kinase that regulates embryonic terminal cell fate in *Drosophila*, is the PTTH receptor (Rewitz et al., 2009) and that the receptor tyrosine kinase is related to PTTH-stimulated ERK phosphorylation in *B. mori* prothoracic glands (Gu et al., 2010).

In addition to PTTH stimulation, recent studies showed that the insulin signaling pathway plays an important role in regulating growth, development, lifespan, as well as metabolic homeostasis (Wu and Brown, 2006). Molecular genetic studies in *Drosophila* revealed that insulin signaling is essential for normal growth, and that it functions as a mediator between nutrition and cell growth (Britton and Edgar, 1998; Britton et al., 2002; Hafen, 2004; Edgar, 2006; Giannakou and Partridge, 2007). Insulin stimulates tyrosine phosphorylation and activates insulin receptor. The insulin receptor transmits a signal via the insulin receptor substrate followed by activation of phosphoinositide 3-kinase (PI3K) (Taniguchi et al., 2006; Liu et al., 2010; Teleman, 2010). PI3K is an important mediator of

* Corresponding author. Fax: +886 42 323 2146.
E-mail address: gu330@mail.nmns.edu.tw (S.-H. Gu).

0965-1748/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2010.12.004

insulin-mediated intracellular signal transduction involving conversion of phosphatidylinositol-3,4,5-triphosphate (PIP_3) by phosphorylation (Engelman et al., 2006; Vanhaesebroeck et al., 2010). PIP_3 phosphorylates and activates Akt, a serine–threonine kinase, also called protein kinase B (PKB). Akt/PKB mediates most of the PI3K-mediated growth-promoting and metabolic actions of insulin through the phosphorylation of several substrates, including other kinases, signaling proteins and transcription factors that affect cell growth, cell cycle entry, and cell survival (Taniguchi et al., 2006; Teleman, 2010).

The role of insulin/PI3K/Akt signaling pathway in size and growth control emerged from studies in *Drosophila*, where loss-of-function mutants of the pathway components were found to display reduction of tissue growth associated with either lethality or severely reduced body size (Teleman, 2010). For example, it has been reported that inhibiting insulin/PI3K signaling phenocopies the cellular and organismal effects of starvation and activating this signaling bypasses the nutritional requirement for cell growth (Britton et al., 2002). In addition, it is documented that ectopic expression of PI3K specifically in the prothoracic glands, accelerates the prothoracic gland's growth, promotes precocious metamorphosis, shortens the larval growth period, and eventually reduces adult size. The increased transcription of at least two ecdysone biosynthesis genes and hence precocious ecdysone synthesis appear to be responsible for precocious metamorphosis (Caldwell et al., 2005; Colombani et al., 2005). Conversely, insulin signaling inhibition in prothoracic glands by expressing either dominant negative PI3K or PTEN, a phosphatase suppressor of insulin signaling pathway, has the opposite effect, delaying the increase in ecdysone synthesis, lengthening the larval growth period and created larger flies (Caldwell et al., 2005; Colombani et al., 2005). It was also reported that increasing insulin signaling by transgene manipulation in the insulin producing cells in *Drosophila* advanced metamorphosis by accelerating ecdysone synthesis (Walkiewicz and Stern, 2009). Recently, our study further showed that bovine insulin stimulates ecdysteroidogenesis by *B. mori* prothoracic glands in vitro through the PI3K/Akt pathway (Gu et al., 2009). However, the direct link from insulin signaling to ecdysteroidogenesis and the ligand that activates insulin signaling are not clear.

In the present study, we investigated the involvement of PI3K/Akt signaling in PTTH-stimulated ecdysteroidogenesis by *B. mori* prothoracic glands. Our results showed that PI3K/Akt signaling is activated by PTTH. Moreover, results indicate that PI3K/Akt and ERK signaling may be two distinct signaling pathways involved in PTTH-stimulated ecdysteroidogenesis in *B. mori*. Thus, the present study, together with the previous demonstration that insulin stimulates silkworm ecdysteroidogenesis through PI3K/Akt signaling, suggests that crosstalk exists in *B. mori* prothoracic glands between insulin and PTTH signaling, which may play a critical role in precisely regulated ecdysteroidogenesis during development.

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-L: 12-D photoperiod. Newly-ecdysed last instar larvae were collected and used for each experiment.

2.2. Reagents and antibodies

Grace's insect cell culture medium was purchased from Gibco (Invitrogen, Carlsbad, CA, USA). LY294002 and wortmannin were purchased from Calbiochem (San Diego, CA, USA). [23, 24-^{3}H] Ecdysone was purchased from New England Nuclear (Boston, MA, USA). Recombinant *B. mori* PTTH was produced by infection of *Spodoptera frugiperda*-SF21 cells with the vWTPTTHM baculovirus as described previously (O'Reilly et al., 1995). The same PTTH as that previously reported (O'Reilly et al., 1995; Gu et al., 2010) was used in the present study. In the present study, the extracellular fluid from cells infected with vWTPTTHM rather than pure PTTH was used as PTTH source, and it was diluted 500 times with medium.

A rabbit polyclonal antibody directed against *Drosophila* phosphorylated Akt (Ser505) and anti-phospho-ERK, anti-total-ERK, and anti-α-tubulin antibodies were 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 prothoracic glands, radioimmunoassay (RIA) of ecdysteroids, and in vivo injection of PTTH

Prothoracic glands from precisely timed larvae were dissected in lepidopteran saline and then incubated for 2 h with gentle shaking in 50 μl of Grace's medium. After incubation, the released ecdysteroids were determined by a RIA according to procedures described in a previous study (Takeda et al., 1986). The assay was calibrated with 20-hydroxyecdysone as the standard. The antiserum has an approximate binding ratio of 3:1 for 20-hydroxyecdysone to ecdysone (Takeda et al., 1986). The detection limit of the RIA was 0.03 ng. To study the in vivo effect on Akt phosphorylation of the prothoracic glands by PTTH, last instar larvae from day 6 were injected with 10 μl saline containing 1 μl of the original PTTH solution. Larvae injected with only 10 μl saline were used as the controls.

2.4. Western blot analysis

Prothoracic glands from precisely staged larvae were homogenized in lysis buffer (10 mM Tris and 0.1% Triton x 100) at 4 °C (Lin and Gu, 2007; Gu et al., 2009). Gland lysates were boiled in an equal volume of sodium dodecylsulfate (SDS) sample buffer for 4 min followed by centrifugation at 15,800×*g* for 3 min to remove any particulate matter. Aliquots of the supernatants were loaded onto either 12% or 10% SDS gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using an Owl (Portsmouth, NH, USA) Bandit™ Tank Electroblotting System, and then washed with Tris-buffered saline (TBS) for 5 min at room temperature. Blots were then blocked at room temperature for 1 h in TBS containing 0.1% Tween 20 (TBST) and 5% (w/v) nonfat powdered dry milk, followed by washing three times for 5 min each with TBST. Blots were incubated overnight at 4 °C with the primary antibody (1:3000) in TBST with 5% bovine serum albumin (BSA). Blots were then washed three times in TBST for 10 min each and further incubated with the horseradish peroxidase-linked second antibody in TBST with 1% BSA. Following three additional washes, the immunoreactivity was visualized by chemiluminescence using Western Lightning Chemiluminescence Reagent *Plus* from PerkinElmer Life Sciences. Films exposed to the chemiluminescent reaction were scanned and quantified using an AlphaImager Imaging System and AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA).

2.5. Data analysis

Results are expressed as the mean + S.E.M. Data for ecdysteroidogenesis were compared by analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. $p < 0.05$ was considered significant.