Insect Biochemistry and Molecular Biology 39 (2009) 171–179

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Insulin stimulates ecdysteroidogenesis by prothoracic glands in the silkworm, *Bombyx mori*

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ARTICLE INFO

Article history:
Received 23 May 2008
Received in revised form
27 October 2008
Accepted 30 October 2008

Keywords:
Bombyx mori
Insulin
Insulin receptor
Ecdysteroidogenesis
Prothoracic gland
PI3K
Signal transduction
In vivo
Akt phosphorylation

ABSTRACT

It is generally accepted that the prothoracicotropic hormone (PTTH) is the stimulator of ecdysteroidogenesis by prothoracic glands in larval insects. In the present study, we investigated activation of ecdysteroidogenesis by bovine insulin in prothoracic glands of the silkworm, *Bombyx mori*. The results showed that the insulin stimulated ecdysteroidogenesis during a long-term incubation period and in a dose-dependent manner. In addition, insulin also stimulated both DNA synthesis and viability of prothoracic glands. Insulin-stimulated ecdysteroidogenesis was blocked by either LY294002 or wortmannin, indicating involvement of the phosphatidylinositol 3-kinase (PI3K) signaling pathway. Activation of ecdysteroidogenesis by insulin appeared to be developmentally regulated. Moreover, in vitro activation of ecdysteroidogenesis of prothoracic glands by insulin was also verified by in vivo experiments: injection of insulin into day 6 last instar larvae greatly increased both hemolymph ecdysteroid levels and ecdysteroidogenesis 24 h after the injection, indicating its possible in vivo function. Phosphorylation of Akt and the insulin receptor was stimulated by insulin, and stimulation of Akt phosphorylation appeared to be PI3K-dependent and developmentally regulated. Insulin did not stimulate extracellular signal-regulated kinase (ERK) signaling of the prothoracic glands. These results suggest that in silkworm prothoracic glands, in addition to the PTTH and an autocrine factor, ecdysteroidogenesis is also stimulated by insulin during development.

1. Introduction

In metazoans, the insulin signaling pathway plays an important role in regulating growth, development, lifespan, as well as metabolic homeostasis (Wu and Brown, 2006). In the past few years, extensive molecular genetic studies in *Drosophila* have 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; Ikeya et al., 2002; Hafen, 2004; Edgar, 2006; Giannakou and Partridge, 2007). When insulin signaling is reduced, by partial loss-of-function mutations in either the insulin receptor (InR) or in genes of the insulin signal transduction pathway, a severe delay in development and a reduction in body size are caused, resulting in small adult flies (Edgar, 2006; Giannakou and Partridge, 2007). By contrast, when insulin signaling is artificially enhanced (by transgene expression), an increase in both growth and body size is observed. More recently, it has been well demonstrated that ectopic expression of phosphatidylinositol 3-kinase (PI3K), an insulin signaling component, specifically in prothoracic glands accelerates growth and creates smaller flies, whereas insulin inhibition in prothoracic glands reduces growth rate and creates larger flies (Colombani et al., 2005). That study further indicated that the observed effects could be attributed to changes in ecdysteroid levels and that the InR-PI3K signaling pathway may specifically activate ecdysteroid production by the prothoracic glands. The effects of PI3K on ecdysteroid production appear to be due to its effect on prothoracic gland size: the expression of PI3K specifically in prothoracic glands increases cell sizes, leading to precocious ecdysteroid release (Colombani et al., 2005). However, it is not clear how insulin signaling is linked to prothoracic gland ecdysteroid production.

By contrast, ecdysteroidogenesis in prothoracic glands is under the control of the prothoracicotropic hormone (PTTH), a neuropeptide produced by the brain (Ishizaki and Suzuki, 1994; Gilbert et al., 2002; Rybczynski, 2005). It was previously demonstrated that PTTH stimulation of ecdysteroid synthesis appears to be mediated by cAMP as an intracellular second messenger in both *Manduca sexta* (Smith et al., 1984, 1985; Gilbert et al., 2002) and *Bombyx mori* (Gu et al., 1996, 1997, 2000). The involvement of Ca^{2+} and extracellular signal-regulated kinase (ERK) in PTTH stimulation of ecdysteroidogenesis has also been well documented (Smith et al., 1985; Gu et al., 1998; Fellner et al., 2005; Lin and Gu, 2007). More

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doi:10.1016/j.ibmb.2008.10.012

recently, it was reported that in addition to the PTTH, an unidentified autocrine factor is also involved in stimulating both prothoracic gland cell growth and ecdysteroidogenesis (Gu, 2006, 2007; Vandersmissen et al., 2007). These results indicate multiple regulatory mechanisms of insect prothoracic gland ecdysteroidogenesis. Although it was previously reported that bovine insulin stimulates ecdysteroid production by ovaries in vitro in both *Aedes aegypti* and *Phormia regina* (Graf et al., 1997; Riehle and Brown, 1999; Manière et al., 2004), it is not clear whether or not insulin can stimulate ecdysteroidogenesis by lepidopteran insect prothoracic glands. It was demonstrated that bombyxin, an insulin-like peptide in *B. mori*, stimulated prothoracic glands isolated from *Samia cynthia*, but not those from *B. mori* (Ishizaki and Suzuki, 1994; Wu and Brown, 2006). In the present study, we investigated activation of ecdysteroidogenesis by bovine insulin in prothoracic glands of the silkworm, *B. mori*. Our results showed that bovine insulin stimulates ecdysteroidogenesis of prothoracic glands both in vitro and in vivo and that this stimulation is PI3K/Akt-dependent.

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 fourth and last instar larvae were collected and used for each experiment, respectively.

2.2. Reagents and antibodies

Bovine insulin and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Grace's insect cell culture medium was purchased from Invitrogen (Carlsbad, CA, USA). LY294002, wortmannin, mpV(pic), and HNMPA-$(AM)_3$ were purchased from Calbiochem (San Diego, CA). [23,24-^{3}H] Ecdysone was purchased from New England Nuclear (Boston, MA, USA). The same recombinant *B. mori* PTTH (rPTTH) as previously reported (O'Reilly et al., 1995; Gu et al., 1998; Chen and Gu, 2006) was used in the present study. Although much time has passed since we obtained it, our study showed that the present rPTTH is stable (Chen and Gu, 2006). Thus in the present study, we used rPTTH as the PTTH source and diluted it 500 times with medium.

Antibodies directed against *Drosophila* phosphorylated Akt (Ser505), mammalian phosphorylated insulin receptor (Tyr1135/1136), anti-phospho-ERK, anti-phospho-mitogen-activated kinase-ERK kinase (MEK) and actin 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 and radioimmunoassay (RIA) of ecdysteroids

Prothoracic glands from precisely timed larvae were dissected under lepidopteran saline (Gu et al., 1995a,b; Gu and Chow, 1996) and then incubated with gentle shaking in 50 μl of Grace's medium. After incubation, the released ecdysteroids were determined by RIA according to procedures described in previous studies (Takeda et al., 1986; Gu et al., 1995a,b; Gu and Chow, 1996). 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. For determination of hemolymph ecdysteroids, hemolymph (100 μl from each animal) was collected and stored at −20 °C before use. Ecdysteroids were extracted from the hemolymph using methanol; after centrifugation, the supernatants were evaporated to dryness. Ecdysteroid activity was measured by an RIA as described above.

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 (Sudhakar and Gopinathan, 2000). Gland lysates were boiled in an equal volume of 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 in TBST with 5% 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. Preparation of brain extracts

To obtain brain extracts, freshly dissected brains from day 7 last instar larvae were homogenized in saline, and the homogenate was immediately heated in a water bath to 100 °C for 2 min to inactivate the peptide-degrading enzymes. After centrifugation (10,000 *g*, for 10 min), the supernatant was sterilized by filtration (through a filter with a pore size of 0.2 μm). The brain extracts were then dissolved in the medium for incubation. For each incubation, approximate 2.5 brain equivalents were used.

2.6. 5-Bromo-2′-deoxyuridine (BrdU) labeling and immunocytochemical staining of BrdU-labeled cells

Proliferation of cells was monitored by in vitro labeling with BrdU (Gu et al., 1999; Gu, 2006). BrdU is incorporated into DNA of dividing cells during the S-phase and is detected using a specific monoclonal antibody (Gratzner, 1982). To study whether or not insulin stimulates prothoracic gland DNA synthesis, similar in vitro labeling with BrdU as previously reported was used (Gu et al., 1999; Gu, 2006).

2.7. MTT colorimetric assay

To study whether or not insulin affects the general metabolic status of the prothoracic glands, the MTT colorimetric assay (Denizot and Lang, 1986; Carmichael et al., 1987; Chow and Gill, 1989) was performed. The assay is dependent on the cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. After 48 incubation, the culture medium was replaced with new Grace's medium and freshly dissolved MTT (stock: 5 mg/ml, in PBS) was aseptically added to a final concentration of 10%. After 3 h incubation, the medium was replaced by 0.1 ml dimethyl sulfoxide to solubilize the colored crystals.