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Signaling of reactive oxygen species in PTTH-stimulated ecdysteroidogenesis in prothoracic glands of the silkworm, *Bombyx mori*

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ABSTRACT

Our previous study demonstrated that mitochondria-derived reactive oxygen species (ROS) generation is involved in prothoracicotropic hormone (PTTH)-stimulated ecdysteroidogenesis in *Bombyx mori* prothoracic glands (PGs). In the present study, we further investigated the mechanism of ROS production and the signaling pathway mediated by ROS. PTTH-stimulated ROS production was markedly attenuated in a Ca^{2+}-free medium. The phospholipase C (PLC) inhibitor, U73122, greatly inhibited PTTH-stimulated ROS production, indicating the involvement of Ca^{2+} and PLC. When the PGs were treated with agents that directly elevate the intracellular Ca^{2+} concentration (either A23187, or the protein kinase C (PKC) activator, phorbol 12-myristate acetate (PMA)), a great increase in ROS production was observed. We further investigated the action mechanism of PTTH-stimulated ROS signaling. Results showed that in the presence of either an antioxidant (N-acetylcysteine, NAC), or the mitochondrial oxidative phosphorylation inhibitors (rotenone, antimycin A, the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and diphenyleneiodonium (DPI)), PTTH-regulated phosphorylation of ERK, 4E-BP, and AMPK was blocked. Treatment with 1 mM of H_2O_2 alone activated the phosphorylation of ERK and 4E-BP, and inhibited AMPK phosphorylation. From these results, we conclude that PTTH-stimulated ROS signaling is Ca^{2+}- and PLC-dependent and that ROS signaling appears to lie upstream of the phosphorylation of ERK, 4E-BP, and AMPK.

1. Introduction

Insect growth, molting, and metamorphosis are initiated and regulated by the steroid hormone ecdysteroids, which are synthesized and secreted in the prothoracic glands (PGs) (De Loof, 2008; Marchal et al., 2010; Smith and Rybczynski, 2012). The mechanism regulating ecdysteroid biosynthesis appears to be a key for clear understanding of how insect metamorphosis is regulated (De Loof, 2008; Marchal et al., 2010; Smith and Rybczynski, 2012; Rewitz et al., 2013; Yamanaka et al., 2013). One of the major stimulators for ecdysteroidogenesis is the prothoracicotropic hormone (PTTH), a neuropeptide, which is produced by brain neurosecretory cells (De Loof, 2008; Marchal et al., 2010; Smith and Rybczynski, 2012; Rewitz et al., 2013; Yamanaka et al., 2013). PTTH activates ecdysteroidogenesis in PGs by binding to its receptor Torso, a receptor tyrosine kinase (Rewitz et al., 2009b, 2013; Smith and Rybczynski, 2012). Downstream of PTTH receptor activation, a complex signaling transduction network is activated (Rewitz et al., 2009a; Marchal et al., 2010; Smith and Rybczynski, 2012). This network includes rapid increase of Ca^{2+} (Fellner et al., 2005; Gu et al., 1998; Birkenbeil and Dedos, 2002), cAMP generation (Smith et al., 1984, 1985; Gu et al., 1996), and activation of protein kinase A (PKA), phospholipase C (PLC), protein kinase C (PKC), p70S6 kinase (S6K), ribosomal protein S6, and tyrosine kinase (Song and Gilbert, 1994, 1995, 1997; Smith et al., 2003; Rybczynski and Gilbert, 2006; Lin and Gu, 2007, 2011; Gu et al., 2010). In addition, phosphorylation of extracellular signal-regulated kinase (ERK) appears to be involved in PTTH-stimulated ecdysteroidogenesis in both *Manduca sexta* and *Bombyx mori* (Rybczynski et al., 2001; Lin and Gu, 2007). Our recent studies further indicate that phosphoinositide 3-kinase (PI3K)/adenosine 5′-monophosphate-activated protein kinase (AMPK)/the target of rapamycin (TOR) signaling is involved in PTTH-stimulated ecdysteroidogenesis in *B. mori* PGs (Gu et al., 2011, 2012, 2013).

For many years, reactive oxygen species (ROS) were viewed as the inevitable but unwanted by-products of cellular metabolism. If the cellular production of ROS overwhelms the cell's antioxidant capacity, damage to cellular constituents, such as lipids, protein, and DNA, may ensue (Veal et al., 2007). An excessive amount of

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ROS is harmful and is considered a causal factor for various pathological conditions including cardiac hypertrophy and insulin resistance (Freeman and Crapo, 1982). However, recent findings suggest that a low concentration of ROS is important for physiological cellular activity (Veal et al., 2007). In mammalian systems, there is growing recognition that ROS may serve as intracellular messenger following receptor activation by a variety of extracellular stimuli including growth factors, cytokines, and hormones (Thannickal and Fanburg, 2000; Veal et al., 2007; Bashan et al., 2009). The mechanism is only now emerging and includes modulation of the thiol proteome, thereby controlling the redox tone that regulates the sensitivity of redox signaling pathways to ROS-dependent activation, inactivation of phosphatases, and activation of tyrosine kinases (Finkel, 1998, 2012; Forman et al., 2010; Collins et al., 2012; Sena and Chandel, 2012; Ray et al., 2012; Liochev, 2013).

In a previous study, we demonstrated that mitochondria-derived ROS signaling is involved in PTTH-stimulated ecdysteroidogenesis in *B. mori* PGs (Hsieh et al., 2013). We found that PTTH rapidly stimulated ROS production and that the antioxidant (N-acetylcysteine (NAC)) and mitochondrial oxidative phosphorylation inhibitors (rotenone, antimycin A, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and diphenyleneiodonium (DPI)) not only greatly inhibited ROS production, but blocked PTTH-stimulated ecdysteroidogenesis. Although these results point to the role of ROS in PTTH signaling network, how ROS are generated and what is the cellular action mechanism of ROS signaling are not clear. In the present study, we demonstrated that ROS production induced by PTTH is Ca^{2+}-and PLC-dependent. We further investigated the modulatory function of redox state on the phosphorylation of ERK, 4E-BP, and AMPK. These results implicate ROS as key modulators in PTTH signaling transduction processes.

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

A23187, the PKC activator (phorbol 12-myristate acetate, PMA), NAC, rotenone, antimycin A, DPI, and FCCP were supplied by Sigma–Aldrich (St. Louis, MO, USA). Grace's insect cell culture medium and 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) were obtained from Molecular Probes/Invitrogen (Carlsbad, 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) was used in the present study. In the present study, extracellular fluid from cells infected with vWTPTTHM was used as the PTTH source, and it was diluted 500 times with medium. Each incubation (50 μl) contained about 0.15 ng PTTH.

Anti-phospho-AMPKα (Thr172), anti-phospho-4E-BP1 (Thr37/46), 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 PGs

PGs from day-5 last instar larvae were dissected in lepidopteran saline (12 mM NaCl, 21 mM KCl, 3 mM $CaCl_2$, 18 mM $MgCl_2$, 9 mM KOH, 170 mM glucose, 5 mM PIPES; pH 6.6). Following dissection, the saline was replaced with fresh medium (± any inhibitors), and a 30-min preincubation period was initiated. After preincubation, glands were rapidly transferred to fresh medium (± experimental materials, such as an inhibitor or PTTH), and then incubated for the indicated times with gentle shaking. In most experiments except for the experiments of Ca^{2+}-free incubation, Grace's medium was used. In the experiments requiring Ca^{2+}-free condition, dissected PGs were preincubated in Ca^{2+}-free saline (i.e. saline without Ca^{2+} but containing 5 mM EGTA) and then transferred to either Ca^{2+}-free saline or saline containing Ca^{2+} (control).

2.4. ROS measurements

ROS generation was monitored by measuring changes in fluorescence resulting from intracellular oxidation of the dye DCFDA (Keller et al., 2004; Hsieh et al., 2010, 2011, 2013). The DCFDA probe enters a cell, is hydrolyzed by cellular esterases, where upon non-fluorescent DCFDA is trapped inside the cell. Subsequent oxidation by ROS yields DCF. Groups of PGs were pre-loaded with DCFDA (20 μM) at 37 °C for 30 min in the dark and washed before a 30-min preincubation period was initiated. After preincubation, the PGs were then incubated for the indicated times with gentle shaking. At the end of incubation the medium was removed, and the DCF fluorescence intensity in each pair of PGs was measured using a fluorescence plate reader (WallacVictor^3 1420 multilabel counter, Perkin Elmer) with an excitation wavelength at 485 nm and emission wavelength of 535 nm. Results are expressed as the percent change from a pair of PGs incubated in control medium.

2.5. Western blot analysis

SDS–PAGE and immunoblotting were performed as previously described (Lin and Gu, 2007; Gu et al., 2011, 2012, 2013). Briefly, the treated or control PGs were homogenized in lysis buffer (10 mM Tris and 0.1% Triton ×100) at 4 °C, then 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 SDS gels. Following electrophoresis, proteins were transferred to polyvinylidenedifluoride (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 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% bovine serum albumin (BSA). Blots were then washed three times in TBST for 10 min each and further incubated with the HRP-linked second antibody in TBST with 1% BSA. Following three additional washes, 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).

3. Results

3.1. Involvement of Ca^{2+} and PLC in PTTH-stimulated ROS production

In a previous study, we demonstrated that PTTH-stimulated ROS production is involved in ecdysteroidogenesis in *B. mori* PGs