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The steroid hormone 20-hydroxyecdysone promotes switching from autophagy to apoptosis by increasing intracellular calcium levels

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ABSTRACT

Autophagy regulates cell survival (or cell death in several cases), whereas apoptosis regulates cell death. However, the relationship between autophagy and apoptosis and the regulative mechanism is unclear. We report that steroid hormone 20-hydroxyecdysone (20E) promotes switching from autophagy to apoptosis by increasing intracellular calcium levels in the midgut of the lepidopteran insect *Helicoverpa armigera*. Autophagy and apoptosis sequentially occurred during midgut programmed cell death under 20E regulation, in which lower concentrations of 20E induced microtubule-associated protein 1 light chain 3—phosphatidylethanolamine (LC3—II, also known as autophagy-related gene 8, ATG8) expression and autophagy. High concentrations of 20E induced cleavage of ATG5 to NtATG5 and pro-caspase-3 to active caspase-3, which led to a switch from autophagy to apoptosis. Blocking autophagy by knockdown of *ATG5*, *ATG7*, or *ATG12*, or with the autophagy inhibitor 3-methyladenine, inhibited 20E-induced autophagy and apoptosis. Blocking apoptosis by using the apoptosis inhibitor Ac-DEVD-CHO did not prevent 20E-induced autophagy, suggesting that apoptosis relies on autophagy. *ATG5* knockdown resulted in abnormal pupation and delayed pupation time. High concentrations of 20E induced high levels of intracellular Ca^{2+}, NtATG5, and active caspase-3, which mediated the switch from autophagy to apoptosis. Blocking 20E-mediated increase of cellular Ca^{2+} caused a decrease of NtATG5 and active caspase-3 and repressed the transformation from autophagy to apoptosis, thereby promoting cell survival. 20E induces an increase in the concentration of intracellular Ca^{2+}, thereby switching autophagic cell survival to apoptotic cell death.

1. Introduction

Autophagy is a process in which cells capture and consume their own cytoplasm and organelles to survive during stress or nutrient shortage (Rabinowitz and White, 2010). The process of autophagy mainly consists of a double-membrane autophagosome and autolysosome formation, which involves two evolutionarily conserved ubiquitin-like conjugation systems, as follows: the Atg12-Atg5 (required for autophagosome membrane formation); and ATG8 (also known as microtubule-associated protein 1 light chain, LC3). LC3 can be divided by cysteine protease and then connected to phosphatidylethanolamine (PE) to form ATG8-PE/LC3-II, which is marked in the double-membrane of autophagosomes and autolysosomes as an indicator of autophagy (Burman and Ktistakis, 2010; Liang, 2010). Apoptosis is characterized by DNA fragmentation and caspase activation; therefore, caspase activity can be used to diagnose apoptosis (Baehrecke, 2005). Caspase-3 is an apoptosis executor that indicates apoptosis (Courtiade et al., 2011). The upregulation of cleaved-caspase-3 level represents apoptosis reinforcement (Torkzadeh-Mahani et al., 2012). ATG5 is cleaved by calcium-dependent proteinase calpain to produce N-terminal ATG5 (NtATG5), which inhibits autophagy and targets NtATG5 to mitochondria, thereby inducing the release of cytochrome *c* to switch autophagy to apoptosis (Pyo et al., 2005).

In addition to autophagic cell survival, autophagic death has been reported in *Drosophila* salivary glands (Berry and Baehrecke,

Abbreviations: Ac-DEVD-CHO, N-Acetyl-Asp-Glu-Val-Asp-CHO; ATG, autophagy related; DAPI, 4-6-diamidino-2-phenylindole dihydrochloride; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; FL, flunarizine dihydrochloride; HaEpi, *Helicoverpa* epidermal cell line; HE, hematoxylinandeosin; LC3, microtubule-associated protein 1 light chain 3; NtATG5, N-terminal ATG5; PYR3, pyrazole compound; RFP, red fluorescent protein; XeC, xectospongin C; 3-MA, *3-Methyladenine*; 20E, 20-hydroxyecdysone.

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2007). Autophagy can also be converted to apoptosis under excessive interleukin-24 stimulation as the knockdown/knockout of autophagy-related genes (ATGs) reduces cell death (Bhutia et al., 2011), whereas apoptosis results in cell death (Zocchi et al., 1998). Inhibition or deficiency of apoptosis protein caspase8 results in excessive autophagy (Yu et al., 2004), and inhibition of autophagy induces apoptosis (Fang et al., 2014), thereby suggesting counteractive roles between autophagy and apoptosis. The relationship among autophagy, apoptosis, and the regulatory mechanisms are complicated and are not completely demonstrated (Tracy and Baehrecke, 2013).

Calcium mobilization and homeostasis in cells are important for a number of cellular functions (Berridge et al., 2000). Cytosolic Ca^{2+} is kept at low levels (10–100 nM), whereas extracellular Ca^{2+} is maintained at much higher levels (Clapham, 2007). In a few cell types, increasing Ca^{2+} can trigger autophagy, whereas increasing Ca^{2+} in others can activate calpain to cleave ATG in order to generate ATG5 to NtATG5, thereby inhibiting autophagy but promoting apoptosis (Friedrich, 2004; Herrero-Martin et al., 2009; Hoyer-Hansen et al., 2007; Pinter and Friedrich, 1988; Pyo et al., 2005). Ca^{2+} could be the key factor in regulating autophagy and apoptosis. However, the regulation role of Ca^{2+} in either autophagy or apoptosis is unclear.

The midgut undergoes remodeling, which involves the degradation of larval midgut and the formation of an imaginal midgut during metamorphosis in insects (Hakim et al., 2010). The degradation of the larval midgut is involved in programmed cell death (PCD); this process provides nutrients for the imaginal midgut formation in lepidoptera (Tettamanti et al., 2007).

The midgut PCD in dipteran *Drosophila* resulted from autophagic cell death but not apoptotic cell death (Denton et al., 2009). *Drosophila* midgut autophagy and apoptosis are both enhanced by steroid hormone 20E (Santhanam et al., 2014). Autophagy and apoptosis sequentially occurred in midgut PCD in lepidopteran *Bombyx* (Franzetti et al., 2012). The steroid hormone 20E promotes midgut PCD (Iga et al., 2010; Manaboon et al., 2009). 20E also promotes autophagy-related and apoptosis-related gene expression (Romanelli et al., 2014). Moreover, 20E can induce an intracellular Ca^{2+} increase to promote apoptosis in the lepidopteran insect *H. armigera* (Cai et al., 2014; Liu et al., 2014; Wang et al., 2016). Therefore, the 20E-induced midgut PCD is a good model for studying the relationship between autophagy and apoptosis.

H. armigera, a *Lepidopteran* insect that is considered a serious agricultural pest, was used as the model for these experiments. High 20E concentration induces high intracellular Ca^{2+} levels, which induces the transformation from autophagy to apoptosis. Higher Ca^{2+} levels regulate ATG5 cleavage to NtATG5 to active caspase-3 for apoptosis. Autophagy maintains cell survival whereas apoptosis results in cell death. The higher concentration of intracellular Ca^{2+} switches autophagic cell survival to apoptotic cell death under 20E induction.

2. Materials and methods

2.1. Animals and cell culture

H. armigera larva were raised on an artificial diet at 28 °C with a dark to light (10:14 h) in the laboratory. The *H. armigera* epidermal cell line (HaEpi) was cultured at 26 °C and was seeded in 4 mL of Grace's medium including 10% inactivated bovine serum without any antibiotics (Shao et al., 2008). All of the experiments were carried out at a density of 5×10^5 cells and were maintained under normal growth conditions for 96 h.

2.2. 20E treatment of HaEpi cells

The HaEpi cells were cultured with 5 μM 20E (Sigma, St Louis, MO, USA) for 1, 6, 24, 48, or 72 h or cultured with 1, 2, 5, 10 μM 20E for 24 h in Grace's medium at 27 °C. The same volume of dimethylsulfoxide (DMSO) was added as a 20E solvent control. The total proteins were extracted from cells with 40 mM Tri-HCl for Western blot assays.

2.3. Western blot

The Bradford method was used to determine the protein concentration (Bradford, 1976). Fifty micrograms of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) and then transferred onto nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in blocking buffer consisting of a 10 mM Tri-buffered saline (TBS) solution and 2–5% fat-free powdered milk. The primary rabbit anti-LC3, ATG5 or Caspase-3 polyclonal antibody was added into the blocking buffer (1:100 diluted) overnight at 4 °C, and then, the secondary rabbit antibody, labeled with alkaline phosphatase, was added at a dilution of 1:10000 in blocking buffer. The Western blot signal was observed in 10 mL of a Tri-buffered saline solution combined with 45 μL of 5% p-nitro-blue tetrazolium chloride (NBT) and 35 μL of 5% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the dark for 10 min. Quantitative analysis of Western bands was performed by Quantity One® software.

2.4. Autophagy detection

The anti-rabbit polyclonal antibody against *H. armigera* LC3 was prepared to detect LC3 by Western blot analysis. *H. armigera* full length LC3 was expressed in *Escherichia coli* by a *PET-30a* plasmid. Two-hundred micrograms of purified LC3 in 1 mL of TBS was mixed with 1 mL of complete Freund's adjuvant and injected into a rabbit subcutaneously to prepare the antibody according to the previous description (Sui et al., 2009). ATG5 antiserum was prepared by the same method. The green fluorescence protein (GFP) was fused with LC3 to produce GFP-LC3-II to indicate the development of autophagy from autophagosomes to autolysosomes by GFP laminating fluorescence at neutral pH in autophagosomes and quenching at acidic pH in autolysosomes (Tasdemir et al., 2008). A RFP-GFP-LC3-His fusion protein was overexpressed for 48 h in HaEpi cells by *pIEx-4-RFP-GFP-LC3-His* reporter plasmid to detect autophagosomes and autolysosomes. A cell-penetrating TAT peptide (TATGGCAGGAAGAAGCGGAGACAGCGACGAAGA) (Zhou et al., 2015) was fused with RFP and LC3 (His-TAT-RFP-LC3-His) and was expressed in *E. coli* by the *pET30a-TAT-RFP-LC3* plasmid. His-TAT-RFP-LC3-His was purified to detect autophagosomes in HaEpi cells. The autophagy inhibitor *3-Methyladenine* (10 μM, 3-MA, NO. 5142-23-4, Gene Operation, USA) was added or injected *as a negative control* in HaEpi cells or the midgut, respectively, to detect the expression of LC3/autophagosomes in HaEpi cells and midgut.

2.5. Immunohistochemistry

The larval midgut was isolated and then treated with 4% paraformaldehyde at 4 °C overnight and gradient-dehydrated. The prepared midgut tissues were embedded in paraffin, cut into 7-μm sections, adhered to gelatin-coated glass slides, and dried at 42 °C overnight. The slides were dewaxed, gradient-rehydrated and then digested with 20 μM proteinase K at 37 °C for 10 min. The slides were blocked in blocking buffer for 30 min at 37 °C and a rabbit anti-*H. armigera* LC3 polyclonal antibody (1: 100) or anti-human, mouse, rat Caspase-3 polyclonal antibody (pAb) (1: 300) (WL01589, Wanleibio,