Ectopic expression of ecdysone oxidase impairs tissue degeneration in Bombyx mori

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Metamorphosis in insects includes a series of programmed tissue histolysis and remodeling processes that are controlled by two major classes of hormones, juvenile hormones and ecdysteroids. Precise pulses of ecdysteroids (the most active ecdysteroid is 20-hydroxyecdysone, 20E), are regulated by both biosynthesis and metabolism. In this study, we show that ecdysone oxidase (EO), a 20E inactivation enzyme, expresses predominantly in the midgut during the early pupal stage in the lepidopteran model insect, Bombyx mori. Depletion of BmEO using the transgenic CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/RNA-guided Cas9 nucleases) system extended the duration of the final instar larval stage. Ubiquitous transgenic overexpression of BmEO using the Gal4/UAS system induced lethality during the larval–pupal transition. When BmEO was specifically overexpressed in the middle silk gland (MSG), degeneration of MSG at the onset of metamorphosis was blocked. Transmission electron microscope and LysoTracker analyses showed that the autophagy pathway in MSG is inhibited by BmEO ectopic expression. Furthermore, RNA-seq analysis revealed that the genes involved in autophagic cell death and the mTOR signal pathway are affected by overexpression of BmEO. Taken together, BmEO functional studies reported here provide insights into ecdysone regulation of tissue degeneration during metamorphosis.

1. Background

Steroid hormones, ecdysteroids (20-hydroxyecdysone, 20E, is the most active form) regulate insect development and metamorphosis. 20E binds to the canonical nuclear receptor complex EcR/USP and regulates many cellular and physiological processes [1–3]. Pulses of 20E are precisely regulated by both synthesis and metabolism pathways. The biosynthesis pathway of 20E has been well documented except for some genes involved in the so-called ‘Black Box’ [4]. By contrast, less is known about 20E metabolism. Although several genes that function in 20E inactivation have been identified, the biological functions of genes involved in this pathway are not well understood [3,5].

One route of 20E inactivation is through 26-hydroxylation to 26-oic acid by the cytochrome P450 CYP18A1 [5–7]. In Drosophila melanogaster, accumulation of 20E after CYP18A1 knockdown repressed the expression of βFtz-F1 and caused pupation defects [5]. By contrast, ubiquitous CYP18A1 overexpression resulted in embryonic lethality and fat body-specific overexpression led to lethality during pupation [5]. Furthermore, CYP18A1 mediated 20E inactivation has been identified in several lepidopteran insects, including Spodoptera littoralis, Manduca sexta and Bombyx mori [8,9]. Distinct from CYP18A1 expression in the peripheral tissues of many insect species, B. mori CYP18A1 is predominantly...
expressed in the middle silk gland (MSG) and transgenic overexpression of BmCYP18A1 leads to lethality during the final instar larval stage [9].

Another major inactivation route of steroid hormone is through 3-epimerization [10–12]. 20E conversion to 3-dehydroecdysone (3DE) can be catalysed by ecdysone oxidase (EO), which could be reversed by 3-dehydroecdysone 3β-reductase [13]. The intermediate, 3DE, is converted to 3-epipeucedone by 3-dehydroecdysone 3α-reductase irreversibly [13,14]. As one of the key enzymes in this pathway, EO was first identified and purified from the dipteran insect Calliphora vicina. The purified protein showed specific 3-dehydroecdysone reductase activity for ecdysone and 20E [15,16]. In several lepidopteran insects and D. melanogaster, EO is predominantly expressed in the midgut and is 20E-responsive [10–12,17]. However, its biological function in insect development and metamorphosis is unknown mainly owing to lack of mutants or transgenic insects overexpressing EO.

Here, we describe the first functional analysis of insect EO by using both loss-of-function and gain-of-function genetic approaches recently developed in the lepidopteran model insect, B. mori [18,19]. We constructed a transgenic CRISPR/Cas9 system to somatically mutate BmEO that caused an extension in the final instar larval stage. By contrast, ubiquitous overexpression of BmEO by using transgenic Gal4/UAS system resulted in lethality during the larval–pupal transition. Furthermore, when BmEO was ectopically expressed in a MSG-specific manner, MSG degeneration at the onset of metamorphosis was severely impaired. Disruption of MSG degeneration by overexpressing BmEO provides an excellent model for understanding the mechanisms of hormonal regulation of tissue degeneration in insects.

2. Material and methods

The detailed information on silkworm strain, cDNA synthesis and qRT-PCR, plasmid construction, germline transformation, RNA-seq, TEM, paraffin embedding, ecdysteroid titer determination and Western blotting is included in the electronic supplementary material, material and methods.

3. Results

(a) Predominant expression of BmEO in the midgut during the early pupal stage

Previous studies reported high EO activity during the early pupal stage in many insects although the functional significance is unknown [13]. We investigated spatial and temporal expression patterns of BmEO in three development stages and 10 tissues using qRT-PCR. The maximum BmEO mRNA levels were detected in the midgut dissected from day 1 pupae (P1, figure 1a). Relatively higher levels of BmEO mRNA were also detected in the fat body (35% of the levels in midgut) and testis (20% of the levels in midgut) dissected from P1 (figure 1a). We further analysed BmEO mRNA levels in the midgut and fat body from day 1 of fifth instar larvae (L5D1) to day 9 of the pupal stage (P9). BmEO mRNA levels in midgut dissected from larvae remained very low and began to increase from day 1 of the prepupal stage (P1P).

During the pupal stages, BmEO mRNA levels in the midgut reached the maximum at P4 and decreased again from P5 to P9 (figure 1b). Although its relative mRNA levels in the fat body showed a similar pattern, the maximum levels reached were only 31.2% of that in the midgut (figure 1b). Abundant BmEO protein was also detected during the early pupal stage (P3 and P4) in midgut (figure 1c). These data suggested that BmEO is predominantly expressed in the midgut during the early pupal stage.

(b) CRISPR/Cas9 mediated-BmEO knockout extended the final instar larval stage

Recently, the CRISPR/Cas9 system has been demonstrated to be effective in targeted gene knockout analysis in D. melanogaster and B. mori [19,20], providing a powerful genetic tool for insect functional genomics. Here, we established a binary transgenic CRISPR/Cas9 system to knockout BmEO somatically. We created a transgenic silkworm line expressing Cas9 ubiquitously under the control of IE1 promoter (IE1-Cas9) and another line expressing the sgRNA targeting BmEO driven by the silkworm U6 promoter (U6-BmEOsgRNA, figure 2a). In heterozygous IE1 > BmEOsgRNA offspring at the target site were detected in all examined animals by PCR analysis (figure 2b), demonstrating that this somatic mutagenesis system was effective. IE1 > BmEOsgRNA animals (60.6%, n = 33) showed larger body size than the control animals and prolonged final instar larval stadium by 24 h (electronic supplementary material, table S1). A significant decrease in BmEO protein levels was also detected in the midgut dissected from P4 BmEO knockout animals (figure 2c). To assess the involvement of BmEO in 20E inactivation, ecdysteroid titers were determined by enzyme immunoassay (EIA). A twofold increase in the ecdysteroid titers was detected in the midgut of BmEO knockout animals on L5D4, when the endogenous ecdysteroid levels are low (figure 2d). We also investigated the mRNA levels of mTOR pathway genes, which are the major regulators of cell growth (figure 2e). Two positive regulators including insulin receptor substrate (IRS) and phosphoinositide-3-kinase (PI3K) were upregulated by 7.4- and 8.7-fold, respectively, in BmEO knockout animals. On the contrary, two negative regulators tuberous sclerosis (TSC1 and TSC2) were downregulated, respectively, to 4.8% and 0.5% of the levels in the control animals (figure 2e).

(c) Ubiquitous overexpression of BmEO disrupted larval–pupal transition

To further investigate BmEO biological functions, it was ubiquitously overexpressed using A3-Cat4 activator. The mRNA levels of BmEO in A3 > BmEO animals were increased by 8.3-fold in the midgut, twofold in the MSG, threefold in the epidermis and fourfold in the fat body when compared to that in the control UAS-BmEO animals on L5D4 (figure 3a). A significant increase in the BmEO protein abundance was also detected in midgut from A3 > BmEO transgenic animals during the wandering stage (figure 3b). Additionally, ecdysteroid levels in A3 > BmEO silkworms decreased by 90% in the midgut and 30% in the haemolymph during the wandering stage (figure 3c). No abnormal phenotype was observed in the transgenic silkworms during embryogenesis and the early larval stages. However, 86% (n = 43) A3 > BmEO animals arrested development prior to pupation (figure 3d). Most of them completed head capsule slippage but failed
to undergo ecdysis, leaving the old cuticle covering the newly formed pupal cuticle (figure 3d). The rest of the 14% transgenic silkworms survived pupation but died during the pupal stage.

(d) Middle silk gland-specific overexpression of BmEO disrupted tissue histolysis

Our previous studies showed that the 20E inactivation enzyme, BmCYP18A1, is predominantly expressed in B. mori MSG [9]. To investigate the functional difference between BmEO and BmCYP18A1, we overexpressed BmEO specifically in MSG using the Ser-Gal4 driver. BmEO mRNA expression increased to more than 1000-fold higher in the MSG of Ser>BmEO animals on L5D4 when compared to its levels in the MSG from the control UAS-BmEO animals (figure 4a). There was no significant increase detected in other five main tissues including midgut, anterior silk gland (ASG), posterior silk gland (PSG), fat body and epidermis. BmEO protein levels also increased in the MSG of Ser>BmEO animals at the wandering stage (figure 4c). Increase in BmEO caused a decrease in the ecdysteroid levels by 72% in the haemolymph and 82% in the MSG of Ser>BmEO animals during the wandering stage (figure 4b).

Almost all (98%, n = 87) Ser>BmEO silkworms successfully completed larval–pupal transition (electronic supplementary material, table S1), showing a bigger size than the control (UAS-BmEO) animals of the same age (figure 4d). Further observation revealed that MSGs were intact in Ser>BmEO pupae at P2, when MSG was degraded and completely disappeared in the control animals (figure 4e). This status was kept to adult stage and the defective MSGs disappeared finally. By contrast, the ASG and the PSG in transgenic animals degraded normally, leaving MSG with caecum at the ends.
Morphological observations showed an enlarged size of MSG on L5D6 and the wandering stage, prior to MSG histolysis in UAS>BmEO animals (figure 4f). During the larval–pupal transition, wild-type (WT) silkworms begin to spin and MSG shrank rapidly in response to the rising ecdysteroid levels. However, MSGs were kept intact until the late pupal stage in the Ser>BmEO silkworms (figure 4f), suggesting that MSG histolysis was inhibited by BmEO overexpression. Although no defects were observed during the pupal–adult metamorphosis, neither female nor male Ser>BmEO moths were able to mate with WT animals and therefore produced no offspring.
Phosphorylates Atg13 and inhibits autophagy initiation [21]. Here, overexpression of Supplementary material, figure S1 BmEO and no significant differences were detected (electronic supplementary material, figure S1 a). Using RNA isolated from MSG from the expression levels of apoptosis-related genes by qRT-PCR the control levels, respectively (figure 5 a). BmEO mRNAs were downregulated (figure 5 b). We also examined the expression levels of apoptosis-related genes by qRT-PCR using RNA isolated from MSG from Ser>BmEO and UAS-BmEO and no significant differences were detected (electronic supplementary material, figure S1b). These data suggest that overexpression of BmEO in MSG may affect only autophagy.

As reported previously, the mTOR Ser/Thr kinase phosphorlylates Atg13 and inhibits autophagy initiation [21]. Here, we also found that the mTOR signalling pathway is among the most downregulated KEGG pathways. Four positively regulated genes including BBX (one of the insulin receptor substrates), PI3K, PDK (3-phosphoinositide dependent protein kinase-1) and Rheb (Ras homologue enriched in brain) were downregulated to 13.5%, 15%, 38% and 42%, respectively (figure 5c). The three negatively regulated genes: TSC1, TSC2 and mTOR (mechanistic target of rapamycin) were upregulated by 5.3, 1.5 and 1.6-fold when compared to the levels in control (figure 5c). Although the RNA-seq results showed no significant change in expression of 20E cascade genes, significant decrease in their expression was detected by qRT-PCR (electronic supplementary material, figure S1a). Among these genes, E75B, USP, E74A and Ftz-F1 mRNA levels decreased to 27%, 9%, 5.3% and 5.4% of their levels in control animals (electronic supplementary material, figure S1a). By contrast, EcRB and HR3 showed similar expression levels as that in the control (UAS-BmEO). Notably, 23 silkworm cuticle genes including 12 with chitin binding domains, which may function as components of the inner cuticle layer, were downregulated significantly in Ser>BmEO when compared to their levels in control animals (electronic supplementary material, figure S1c).

**Figure 3.** Ubiquitous overexpression of BmEO caused an arrest in metamorphosis during larval–pupal transition. (a) BmEO mRNA levels increased to 8.3-fold in midgut, two-fold in MSG, threefold in the epidermis and fourfold in fat body when expressed under the control of actin 3 promoter at L5D4. Three individual biological replicates were used in qRT-PCR and the BmEO mRNA levels were normalized to silkworm Bmmp49. (b) BmEO protein showed an increase in the midgut at the wandering stage. (d) Ecdysteroid levels decreased to 10% in the midgut and 70% in haemolymph at the wandering stage. Three individuals were used to extract ecdysteroid and measured by EIA assay. (d) Actin-driven BmEO (A3>BmEO) overexpression caused a metamorphosis arrest during larval–pupal transition. The lower panel is the control UAS-BmEO pupa and upper three are A3>BmEO animals. The asterisks in (a,c) indicate statistical significance (p < 0.05). (Online version in colour.)

(e) Autophagy and mTOR signalling pathway were affected in Ser>BmEO animals

In order to understand the molecular mechanisms underlying the phenotypes observed, MSGs from Ser>BmEO and UAS-BmEO animals were dissected from PP2 (when MSG histolysis is initiated in WT silkworms) and subjected to RNA-seq analysis. Among 832 differentially expressed genes (DEGs) identified, 454 genes including BmEO were upregulated and 378 were downregulated (figure 5a). KEGG enrichment analysis revealed that autophagy and mTOR signalling were in the top five downregulated pathways (figure 5b). As confirmed by qRT-PCR, 13 silkworm Atg genes were downregulated in MSG of Ser>BmEO transgenic silkworms. Among them, Atg1, Atg6 and Atg11 decreased to 6.7%, 9.1% and 6.9% of the control levels, respectively (figure 5c). We also examined the expression levels of apoptosis-related genes by qRT-PCR using RNA isolated from MSG from Ser>BmEO and UAS-BmEO and no significant differences were detected (electronic supplementary material, figure S1b). These data suggest that overexpression of BmEO in MSG may affect only autophagy.

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Middle silk gland-specific *BmEO* overexpression inhibited autophagy in the silk gland

Silkworm silk gland was considered as the homologous tissue of *D. melanogaster* salivary gland which undergoes histolysis triggered by programmed cell death (PCD). To determine whether the defects in MSG degradation could be attributed to disruption of autophagy identified by RNA-seq, we performed transmission electron microscopy (TEM) and LysoTracker staining on the tissues dissected from PP2 animals. TEM showed a large number of autophagic structures in MSGs (figure 6a), which encompassed cytoplasmic components and organelles.
in the double membranes (inset is the enlargement of the granule indicated by green triangle) in MSG of WT animals. However, a lower number and smaller autophagic structures were observed in the MSG of Ser>BmEO animals (figure 6a). Interestingly, autophagy was inhibited both in the fat body (figure 6b,b') and the midgut (figure 6c,c'). This is probably an indirect effect caused by circulating ecdysteroids. LysoTracker staining of MSG sections dissected from PP2 Ser>BmEO animals showed a thinner outer silk gland cell layer when compared with the MSG of UAS-BmEO control animals.
4. Discussion

(a) BmEO mediates midgut-specific 20E inactivation

The current study confirmed midgut-specific expression of BmEO in B. mori, being consistent with its expression in other insect species studied [10–12,17], and revealed its highest expression during the early pupal stage, P4. We employed loss-of-function, gain-of-function, RNA-seq and histological methods to uncover its biological functions in B. mori.

Then employing the CRISPR/Cas9 system, we successfully induced mutagenesis at the BmEO loci, thus providing a promising approach for loss-of-function analysis. Depletion of BmEO did not cause significant changes in silkworm development except for a 24 h extension of the final instar larval stage. By contrast, overexpression of BmEO ubiquitously using the Gal4/UAS transgenic approach induced lethality during the larval–pupal transition. However, ubiquitous overexpression of another 20E inactivation enzyme, MSG-specifically expressed BmCYP18A1, induced larval lethality during the early stages of final larval instar, earlier than that observed in BmEO overexpressed animals [9]. Considering their tissue-specificity, we also ectopically expressed BmEO in MSG, where no BmEO expression is observed in WT animals. Interestingly, a completely different phenotype was observed in Ser>BmEO animals in which MSG developed to a larger size and did not degenerate right after larval–pupal transition. However, MSG-specific overexpression of BmCYP18A1 led to silk gland growth arrestment during the final larval instar [9]. These data suggest temporal and spatial differences in 20E inactivation by BmEO and BmCYP18A1 may go through different routes. Since 3-epimerization catalysed by EO is reversible, inactivation of 20E by BmEO may be less drastic than by BmCYP18A1.

Drosophila melanogaster tissues show a tissue-specific 20E response pattern during larval–pupal metamorphosis [22]. Larval midgut cells begin to undergo autophagy during puparium formation, when an increase in 20E levels occurs. However, the salivary gland cells begin histolysis only during the pre-pupal stage when the 20E levels increase again [22,23]. These data suggest that different tissues respond differently to circulating ecdysteroids. It has been

Figure 6. Decrease in autophagic structures in the Ser>BmEO MSGs examined by the TEM and LysoTracker staining. (a) Significant levels of autophagy observed in MSG from UAS-BmEO transgenic silkworms at PP2. The inset is the enlargement of the double membrane structure indicated by the green triangle. (a’) Less autophagic structures were observed in the Ser>BmEO MSGs. (a”) LysoTracker staining (X40) of paraffin sections of MSG from UAS-BmEO silkworms. Green triangles show the positive staining structures in the silk gland cells of MSGs. (a’’) Decrease in autophagy levels detected in MSGs of the Ser>BmEO silkworms. Thinner silk gland cell layer (the outer layer of MSG) was observed. The second and third rows showed the results from the midgut and fat body, respectively. At least three silkworms were used for each assay. Scale bars are indicated in the figures. (Online version in colour.)

(figure 6a”). Furthermore, autophagic signals in both fat body (figure 6b”,b’’) and midgut (figure 6c”,c’’) showed a significant decrease.
shown that members of 20E genetic circuits are expressed in different temporal and spatial patterns [24,25]. Differences in transcription responses have also been observed between *Drosophila* Kc167 and salivary gland cells [26]. Tissue-specificity of 20E inactivation observed in *B. mori* suggests that the tissue-specific differences in ecdysteroid response may be mediated in part by the differential expression of ecdysteroid inactivation enzymes.

(b) 20E triggered autophagy is required for tissue histolysis in *Bombyx mori*

Studies on *D. melanogaster* salivary glands revealed that insect tissue histolysis is caused by suppression of IIS/TOR pathway and activation of PCD [27–29]. Being the homologous organ of the *D. melanogaster* salivary gland, *B. mori* silk gland is also a larval-specific tissue and undergoes degeneration during larval–pupal metamorphosis [30], accompanied by nucleus pyknosis, cell detachment and membrane blebbing [31]. In addition, the markers of PCD, increase in vacuoles, acid phosphatase activities and caspase-3 activity have been detected in the degenerating silk glands. These data suggest that silk gland histolysis is a result of both apoptosis and autophagy.

Since 20E is one of the stimuli for insect metamorphosis, direct connections between 20E and PCD have been investigated in insects [32–34]. In silkworms, injection of the 20E into the feeding silkworms increased expression of *Atg* genes and mutation of *EcR* resulted in an inhibition of autophagy [35]. Here, we found that overexpression of *BmEO* in MSG inhibited autophagy. qRT-PCR (figure 5c), TEM and LysoTracker staining (figure 6) showed that the autophagy signalling is repressed in *Ser>BmEO* animals. By contrast, no significant differences were detected between *Ser>BmEO* and control animals in the expression of genes involved in apoptosis (electronic supplementary material, figure S1b). Thus, the autophagy pathway may be the main pathway that was suppressed by EO ectopic expression and subsequent 20E decrease in MSG.

Here, we provide, to our knowledge, the first report on insect EO biological function in steroid hormone inactivation. Midgut-specific expression of *BmEO*, along with MSG-specific expression of *BmCYPIA1AI* described in our previous study, revealed spatial and temporal differences in insect steroid hormone inactivation pathways. Additionally, the transgenic CRISPR/Cas9 system used in the current study is a promising approach for somatic mutagenesis analysis for learning functions of genes especially in insects where RNAi is inefficient.

Data accessibility. The RNA-seq raw data was deposited on NCBi SRA database with accession no. SRP052024.

Authors’ contributions. Z.L. performed the experiments, collected and analysed data and wrote the paper; L.Y. performed the microinjection and data collection; B.Z., L.L., J.X., X.C. and Z.Z. participated in the data analysis; S.P, Y.H. and A.T designed the experiment, analysed data and wrote the manuscript.

Competing interests. We declare we have no competing interests.

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