Article

Developmental Regulation of Glycolysis by 20-hydroxyecdysone and Juvenile Hormone in Fat Body Tissues of the Silkworm, *Bombyx mori*

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20-Hydroxyecdysone (20E) and juvenile hormone (JH) control a variety of physiological events during insect development and metamorphosis. To understand how 20E and JH developmentally regulate energy metabolism in insects, we performed a genome-wide microarray analysis of fat body tissues isolated from the silkworm, *Bombyx mori*. Many genes involved in energy metabolism, including genes in the glycolytic pathway, were down-regulated during molting and pupation, when 20E levels are high. Notably, 20E treatment exhibited inhibitory effects on key glycolytic enzyme mRNA levels and activities, and RNA interference of the 20E receptor *EcR–USP* had the opposite effects to 20E treatment. Meanwhile, JH treatment stimulated both mRNA levels and activities of the key glycolytic enzymes, presumably via antagonizing the 20E action. Taken together, we conclude that 20E acts as a general blocker for glycolysis in the *Bombyx* fat body during molting and pupation, whereas the physiological role of JH is contrast with 20E during molting.

Keywords: 20-hydroxyecdysone, juvenile hormone, glycolysis, energy metabolism, fat body, *Bombyx mori*

Introduction

Molting and metamorphosis in insects are coordinately regulated by the molting hormone 20-hydroxyecdysone (20E) and juvenile hormone (JH). Overall, 20E orchestrates the molting process, while JH determines the nature of the molt. In the presence of JH, 20E directs larval molting; whereas in the absence of JH, 20E directs larval-pupal-adult metamorphosis (Riddiford, 1994, 2008). At the molecular level, for example, JH prevents 20E-induced programmed cell death by suppressing the 20E-triggered transcriptional cascade and modulating mRNA levels of several caspase genes (Wu et al., 2006; Liu et al., 2009a).

Insect fat body, analogous to vertebrate adipose tissue and liver, is the main organ involved in energy metabolism. During the feeding stages, insects store glycogen and triglycerides as energy reserves in the fat body. In response to energy demands, insects release trehalose and diglycerides from the fat body to hemolymph for utilization (Hoshizaki, 2005; Arrese and Soulages, 2009; Liu et al., 2009b). In mammals, nuclear receptors, such as FXR, LXR, RXR and PPAR, play essential roles in controlling energy metabolism (Desvergne, 2007). In insects, the 20E receptor complex contains two nuclear receptor molecules, EcR and USP, which are homologues to mammalian FXR/LXR and RXR, respectively (King-Jones and Thummel, 2005). It has been long thought that 20E and JH play important roles in regulating energy metabolism in insects, but it was not until the last decade that genomic and genetic studies in the fruit fly, *Drosophila melanogaster*, have added credibility. Using whole body microarray analyses during *Drosophila* larval-pupal metamorphosis, a large number of genes related to energy metabolism. Nine genes in the glycolytic pathway were observed to be down-regulated during the late larval 20E pulse (White et al., 1999). By using *in vitro* tissue culture with 20E as well as a heat-induced EcR RNAi strain (*hs-EcR–RNAi*), microarray analysis revealed that one-third of all genes in the fly genome are regulated by 20E–EcR–USP, either directly or indirectly (Beckstead et al., 2005). Importantly, many genes induced upon starvation (Zinke et al., 2002) are also regulated by 20E treatment and EcR RNAi (Beckstead et al., 2005). On the other hand, apart from a detailed study by Truman et al. (2006) showing that JH is required to couple imaginal disc formation with nutrition in the tobacco hornworm, *Manduca sexta*, very little is known about how JH regulates energy metabolism.
The silkworm, *Bombyx mori*, is one of the best models to study insect physiology and biochemistry. To understand how 20E and JH developmentally regulate energy metabolism in insects, we performed a genome-wide microarray analysis of the *Bombyx* fat body, during three developmental stages of animals displaying different 20E and JH levels. Many genes involved in energy metabolism, including genes in the glycolytic pathway, were down-regulated during molting and pupation, when 20E levels are high. Hormone treatment and RNA interference (RNAi) experiments demonstrate that 20E acts as a general blocker for glycolysis in the *Bombyx* fat body during molting and pupation, whereas the physiological role of JH is in contrast with 20E during molting. Considering that 20E reduces food consumption resulting in *Bombyx* fat body lipolysis during molting and pupation (Wang et al., 2010), we assume that, during molting and pupation when 20E levels are high, 20E acts as a general blocker for glycolysis in the fat body but activates its lipolysis to meet physiological and developmental requirements to provide energy resources.

**Results**

**Differentially expressed genes related to energy metabolism revealed by microarray analyses**

The genome map (Xia et al., 2004; The International Silkworm Genome Consortium, 2008) and an oligonucleotide microarray (Xia et al., 2007) allowed us to investigate how 20E and JH developmentally regulate gene expression in the *Bombyx* fat body at the whole-genome scale (Unpublished data). In this insect species, both 20E and JH levels are high during the final larval molt (4M) but absent (or extremely low) during the feeding stage of fifth instar (5F), while 20E level is high and JH level is low at the prepupal stage (PP) (Kinjoh et al., 2007; Muramatsu et al., 2008). Since both levels of 20E and JH vary dramatically from 4M to 5F to PP, using microarray, we compared gene expression profiles between 4M and 5F as well as between 5F and PP to understand how the two hormones regulate fat body physiological functions.

The microarray analysis revealed two sets of differentially expressed genes with particular interests. The first interesting set of differentially expressed genes is involved in innate immunity (Unpublished data). The second interesting set is involved in energy metabolism. At both 4M and PP, 20E levels are high. This suggested that the second set of differentially expressed genes (511 genes in total), which were down-regulated at both 4M and PP, were possibly inhibited by 20E. Pronouncedly, about two-thirds of the function-defined genes were involved in energy metabolism. Overall, these genes were critical for most anabolic and catabolic pathways, including the reductive carboxylate cycle, glycolysis, citrate cycle, pentose phosphate pathway and glycogen biosynthesis and degradation. However, the down-regulated genes at 4M were somehow more in number than those at PP in every pathway (Figure 1A). These data imply that 20E possibly inhibits most aspects of energy metabolism in the *Bombyx* fat body during 4M and PP.

We are particularly interested in the genes encoding the glycolytic enzymes. One hundred percent and 80% of these genes were down-regulated at 4M and PP, respectively (Figure 1A). In comparison to 5F, most genes were down-regulated by 30% or even lower at 4M and PP (Figure 1B). In the following studies, we will focus on investigating how 20E and JH developmentally regulate glycolysis in the *Bombyx* fat body.

**Developmental changes of key glycolytic enzyme mRNA levels and activities**

Glycolysis is a central metabolic pathway in all organisms. The first stage of glycolysis is the core module that converts...
glucose into pyruvate and the second stage is metabolism of pyruvate to lactate or ethanol (Supplemental Figure S1). The glycolytic pathway include multiple genes, most of which were reproducibly detected in each chip. Since the microarray data suggest that glycolysis is decreased by 20E during molting and pupation, the mRNA expression profiles of nine genes, which showed comparatively high expression levels in the chips, in the glycolytic pathway were measured from day 2 of fourth instar to day 2 of PP. The nine genes included glucose-1-phosphatase (G1P), hexokinase (HK), glucose-6-phosphate isomerase (G6Pi), 6-phosphofructokinase (6PFK), fructose 1,6-bisphosphate aldolase (FDPALD), phosphopyruvate hydratase (PPH), pyruvate kinase (PK), lactate dehydrogenase (LDH) and pyruvate dehydrogenase E1 component beta (PDHE1β). In general, the mRNA level for each of these genes was high during the feeding stages of both fourth and fifth instars and low at both 4M and PP, when 20E levels are high. Exceptions were found in the mRNA levels of LDH and 6PFK which were elevated at PP (Figure 2A).

The developmental profiles of activities of three key glycolytic enzymes, including HK, 6PFK and FDPALD, were then measured. Low enzyme activities were detected at 4M and PP, with an additional reduction appeared on days 4–6 of fifth instar (Figure 2B). Overall, the developmental change profiles of enzyme activities were similar to but less dramatic than the corresponding changes of gene expression levels. The developmental profiles are consistent with the microarray data suggesting that 20E inhibits glycolysis during molting and pupation.

### Decrease of key glycolytic enzyme mRNA levels and activities by 20E

To provide evidences in support of the above hypothesis, 20E was injected into day 2 of fifth instar larvae and key glycolytic enzyme mRNA levels and activities measured in the fat body 6 h after 20E injection. As expected, the 20E-triggered transcriptional cascade, including expression levels of the 20E primary response genes E75B and Br-C, was significantly enhanced.
Remarkably, mRNA levels of eight metabolic genes were significantly down-regulated by 20E injection. One exception was the 6PFK mRNA level which was not altered by 20E (Figure 3A). Meanwhile, enzyme activities of HK and FDPALD were significantly decreased by 20E injection whereas the decrease of 6PFK activity was little (Figure 3B). The 20E treatment experiments demonstrate that 20E is a general blocker for glycolysis.

**Increase of key glycolytic enzyme mRNA levels and activities by EcR–USP RNAi**

20E acts via its receptor complex EcR–USP to trigger physiological and developmental events (Riddiford et al., 2000). In the previous study, we have shown that EcR and/or USP dsRNA treatment resulted in significant prepupal or pupal death phenotypes and a dramatic decrease of the 20E transcriptional cascade, including expression levels of EcR or USP, as well as E75B and Br-C. EcR and USP RNAi exhibited similar phenotypic and molecular effects, although USP RNAi is a little more effective than EcR RNAi (Unpublished data). Key glycolytic enzyme mRNA levels and activities were then determined after USP RNAi treatment. First, mRNA levels of five glycolytic genes (G1P, FDPALD, PPH, LDH and PDHE1β) were significantly increased and those of two genes (G6Pi and PK) were not altered. In contrast, HK and 6PFK mRNA levels were significantly decreased (Figure 4A). Concurrently, USP RNAi treatment had a significant effect on increasing 6PFK activity but no obvious effects on HK and FDPALD activities (Figure 4B). Conclusively, the effects on glycolysis by USP RNAi are normally opposite to 20E injection.

**Increase of key glycolytic enzymes’ mRNA levels and activities by JH**

It has been long believed that the status quo action of JH is to antagonize 20E action (Riddiford, 1994, 2008). To verify if this is also the case in regulating glycolysis, larvae at the early wandering (EW) stage were topically applied with JH analogue (JHA, methoprene), which was able to effectively prevent the 20E-triggered transcriptional cascade, including expression levels of E75B and Br-C, in the Bombyx fat body at this

![Figure 3 Down-regulation of glycolytic mRNA levels and enzyme activities by 20E. (A) Down-regulation of glycolytic mRNA levels by 20E. Ten animals were used for each group and five biological replicates were conducted in all the hormone treatment experiments. (B) Decrease of glycolytic enzyme activities by 20E with the exception of 6PFK activity. G1P, glucose-1-phosphatase; HK, hexokinase; G6Pi, glucose-6-phosphate isomerase; 6PFK, 6-phosphofructokinase; FDPALD, fructose 1,6-bisphosphate aldolase; PPH, phosphopyruvate hydratase; PK, pyruvate kinase; LDH, lactate dehydrogenase; PDHE1β, pyruvate dehydrogenase E1 component beta. Black: control; gray experimental. Data were represented as mean ± SD. * P < 0.05; ** P < 0.01.](image-url)
developmental stage (Unpublished data). Key glycolytic gene mRNA levels and enzyme activities in the fat body were measured 6 h after JHA treatment. The mRNA levels of five genes (HK, G6P1, glucose-6-phosphate isomerase; 6PFK, 6-phosphofructokinase; FDPALD, fructose 1,6-bisphosphate aldolase; PPH, phosphopyruvate hydratase; PK, pyruvate kinase; LDH, lactate dehydrogenase; PDHE1β, pyruvate dehydrogenase E1 component beta. Black: control; gray: experimental. Data were represented as mean ± SD. * P < 0.05; ** P < 0.01.

Figure 4 Up-regulation of both glycolytic mRNA levels and enzyme activities by USP RNAi. (A) Up-regulation of glycolytic mRNA levels by USP RNAi except mRNA levels of HK and 6PFK. (B) Increase of 6PFK activity by USP RNAi. G1P, glucose-1-phosphatase; HK, hexokinase; G6P1, glucose-6-phosphate isomerase; 6PFK, 6-phosphofructokinase; FDPALD, fructose 1,6-bisphosphate aldolase; PPH, phosphopyruvate hydratase; PK, pyruvate kinase; LDH, lactate dehydrogenase; PDHE1β, pyruvate dehydrogenase E1 component beta. Black: control; gray: experimental. Data were represented as mean ± SD. * P < 0.05; ** P < 0.01.

developmental stage (Unpublished data). Key glycolytic gene mRNA levels and enzyme activities in the fat body were measured 6 h after JHA treatment. The mRNA levels of five genes (HK, G6P1, PK, LDH and PDHE1β) were significantly up-regulated by JHA treatment, although G1P, 6PFK and FDPALD mRNA levels were not altered by JHA. PPH mRNA level was down-regulated by JHA treatment (Figure 5A). All the key glycolytic enzyme activities were found to be significantly or slightly increased by JHA treatment (Figure 5B). The JHA treatment experiments strongly suggest that JH activates glycolysis via antagonizing the 20E action.

Combining the microarray, developmental changes, hormone treatments and RNAi results together, we conclude that 20E has inhibitory effects on glycolytic gene mRNA levels and activities through its receptor EcR and USP, although the physiological role of JH is in contrast with 20E.

Developmental changes of glycogen content in the fat body

In the fat body, glucose can be either mobilized via glycolysis or stored as glycogen (Liu et al., 2009b). Microarray analyses showed that most genes in the glycogen biosynthesis and degradation pathway were also down-regulated at 4M and PP (Figure 1A). To understand if 20E and JH are also involved in glycogen metabolism, we analyzed the developmental changes of glycogen content in the fat body. Estimated by staining (Figure 6) or measured by the Anthonre–Sulfuric Acid method (Figure 7), glycogen storage in the fat body appeared to be extremely low at 4M and PP, and increased gradually with feeding at both fourth and fifth instar but decreased right before molting or wandering. The developmental changes of glycolysis or glycogen content agree with the hypothesis that 20E is a general blocker for energy metabolism in the Bombyx fat body during molting and puation.

Discussion

It has been long thought that 20E and JH play important roles in regulating energy metabolism in insects. During the last decade, a couple of genomic and genetic studies in Drosophila reveal that 20E down-regulates mRNA levels of many genes involved in energy metabolism at the onset of metamorphosis (White et al., 1999) via EcR–USP (Beckstead et al., 2005). They assumed that
some tissues, such as midgut and fat body, are responding to the initiation of metamorphosis by tempering their metabolic activity (White et al., 1999), but later studies showed that the midgut is not the responsible tissue (Li and White, 2003). Although lacking measured enzymatic activities, they proposed that 20E–EcR–USP played a central role in controlling metabolic responses at pupariation, directing the change from a feeding growing larva to an immobile non-feeding pupa (Beckstead et al., 2005).

In this paper, we provided evidences for these hypotheses and proved that fat body was the responding tissue that dramatically tempers metabolic activity in response to 20E-triggered molting and pupation. Pronouncedly, our microarray data revealed that about two-thirds of the down-regulated genes at 4M and PP were involved in energy metabolism, which are critical for most anabolic and catabolic pathways (Figure 1A). We then carefully studied how 20E and JH regulate glycolysis in the Bombyx fat body. Developmental profiles showed that mRNA levels and activities of key enzymes involved in glycolysis were significantly decreased at 4M and PP, when 20E levels are high (Figure 2).

Most importantly, by using hormone treatment and RNAi, we have demonstrated that 20E was a general blocker for glycolysis at 4M and PP (Figure 3) via its receptor complex EcR–USP (Figure 4) and the physiological role of JH is in contrast to 20E at 4M (Figure 5). In addition, the developmental profile of glycogen content in the fat body implies that 20E was also a general blocker for glycogen metabolism (Figures 6 and 7). Taken together, we believe that the fat body is a major organ responding to 20E by tempering its metabolic activity, directing the change from a feeding growing larva to an immobile stage—molting or pupation.

It is necessary to note that the results obtained from 20E treatment (Figure 3) were normally, but not perfectly, opposite to the results from USP RNAi (Figure 4). For example, contrary to the prediction, mRNA levels of HK and G6FK were decreased by USP RNAi. A similar phenomenon was also observed in Drosophila, in which not all 20E response genes can be down-regulated by EcR RNAi (Beckstead et al., 2005). Although it is unlikely, we cannot exclude the possibility that 20E regulates gene expression via an unknown pathway, such as through a membrane receptor. Even in the same RNAi experiments, changes of mRNA levels did not exactly match changes of enzyme activities (Figure 4). For example, although mRNA level of HK was decreased by USP RNAi, HK activity was increased by USP RNAi. This could be due to post-transcription regulation of HK by other factors. Another possibility is that HK activity can be increased by its available substrate. The effects of JH on glycolysis (Figure 5) were almost...
opposite to that of 20E (Figure 3). One exception is the mRNA level of PPH. We assume that the major role of JH during larval molting is to antagonize 20E action. However, it is still possible that JH may function alone independent of 20E, although 20E indeed normally down-regulates most genes involved in energy metabolism (White et al., 1999; Beckstead et al., 2005; Figure 1A), such as 20E-inhibited glycolysis, as is totally different from 20E-activated lipolysis. In a previous study, we have shown that 20E does not directly induce fat body lipolysis in Bombyx. Instead, 20E reduces food consumption via an unidentified tissue(s) (such as brain or midgut) and thus induces starvation resulting in fat body lipolysis by the lipase Brummer during molting and pupation in Bombyx (Wang et al., 2010). On the basis of these two studies, we hypothesize that 20E acts through two different mechanisms to regulate glycolysis and lipolysis in the Bombyx fat body during molting and pupation. Fat body is highlighted as yellow.

**Figure 6** The developmental profiles of glycogen content in Bombyx fat body. The developmental profile of glycogen content in Bombyx fat body from day 2 of fourth instar to day 2 of prepupal stage (PP). The staining method was used for estimation. 4L-1D, day 1 of fourth instar; 4L-3D, day 3 of fourth instar; 4M, the final larval molt; 5L-1D, day 1 of fifth instar; 5L-4D, day 4 of fifth instar; 5L-6D, day 6 of fifth instar; 5L-7D, day 7 of fifth instar; W, the early wandering; PP, the prepupal stage.

**Figure 7** The developmental profile of glycogen content in Bombyx fat body. The Anthrone–Sulfuric Acid method was used for determination. Samples from days 3 and 4 of fourth instar were pooled.

**Figure 8** A proposed model shows that 20E acts through two different mechanisms to regulate glycolysis and lipolysis in the Bombyx fat body during molting and pupation. Fat body is highlighted as yellow.
and thus to induce starvation, which eventually results in lipolysis in the fat body. This process takes 12–24 h. We assume that 20E-induced lipolysis in the fat body might be very important to meet the physiological and developmental requirements for providing energy resource during these critical non-feeding stages, such as molting and pupation, since 20E already blocks the basic metabolism.

Materials and methods

Animals

Bombyx larvae (Nistari) were provided by The Sericultural Research Institute, Chinese Academy of Agricultural Sciences. They were reared with fresh mulberry leaves in the laboratory at 25°C under 14 h light/10 h dark cycles (Liu et al., 2010).

Microarray

The details of the microarray analysis were performed according to the standard process of microarray. The microarray data have been submitted to ArrayExpress and the Gene Expression Omnibus (GEO).

Hormone treatments

Day 2 of 5F (48 h after the first feeding of the fifth instar larvae) was chosen for 20E injection (Sigma Aldrich, USA; 3 μg/larva) and the controls were injected with the same volume of control solvent. At this stage, hemolymph 20E levels were low and the fat body was sensitive to 20E. Twelve hours after the beginning of EW was chosen for topical application of methoprene (Dr Ehrenstorfer GmbH, Germany; 15 μg/larva). At this stage, 20E level just began to rise and the fat body was sensitive to JH. Six hours after hormone treatment hemolymph was collected, and then larvae were sacrificed to dissect fat body tissues for qPCR analysis or measuring enzyme activities. Ten animals were used for each group and five biological replicates were conducted.

RNA interference

Double-stranded RNA (dsRNA) of Bombyx EcR and USP (Swevers et al., 1996) were generated using the T7 RiboMAX™ Express RNAI system (Promega, USA) according to the manufacturer’s instruction. In preliminary studies, we have confirmed that, as a control, ddH₂O is not different from GFP dsRNA. At the beginning of EW, each individual larva was injected with 5 μl of ddH₂O, EcR dsRNA (5 μg), USP dsRNA (5 μg) or EcR (5 μg) and USP (5 μg) dsRNA. At this stage, the Bombyx larvae were sensitive to RNAI treatments. Twenty-four hours after RNAI treatment, the larvae were sacrificed for further measurements as described above. Thirty animals were used for each group and three biological replicates were conducted.

Quantitative real-time PCR

For qPCR analysis, total RNA was extracted from larval fat body tissues of different developmental stages and used for quantitative real-time PCR (qPCR) analysis as previously described (Liu et al., 2010). Bombyx mori RP49 was chosen as a reference gene. qPCR primers and other primers used in this paper are listed (Supplemental Table S1).

Measurements of enzyme activities of HK, 6PKF and FDPALD

Larval fat body tissues were homogenized and extracted for total protein. According to the manufacturer’s instructions (GenMed Scientific, USA), total protein content as well as enzyme activities of HK, 6PKF and FDPALD were measured using a Multiskan Flash Microplate Reader (Thermo Fisher Scientific, USA).

Measurement of glycogen levels in the fat body

Fat body glycogen was extracted by a digestion in 30% KOH followed by ethanol precipitation. After centrifugation, the pellet was resolved in ddH₂O. The amount of glycogen was then determined by the Anthrone–Sulfuric Acid method. In parallel, the glycogen level in the fat body was also estimated using a staining method with a commercial kit (GenMed Scientific).

Statistical analyses

Student’s t-test was used for statistics analyses.

Supplementary data

Supplementary data for this article are available online at http://jmbc.oxfordjournals.org.

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References


Hormonal regulation of *Bombyx* glycolysis


