MOLECULAR BASIS OF ENDOCRINE CONTROL OF INSECT MOLTING AND METAMORPHOSIS

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Received: December 18, 2011
Accepted: December 27, 2011

ABSTRACT

Insect molting and metamorphosis are regulated by two major insect hormones, the juvenile hormone (JH) and ecdysteroids. Ecdysteroids initiate the molting process, whereas JH dictates the character of a molt. At the critical period of ecdysteroid rise, the presence or absence of JH directs larval-larval molting or metamorphosis. Ecdysteroid after binding with its receptor activates the expression of many downstream genes such as E75 and broad, also known as transcription factors. JH modulates the ecdysteroid action by altering the expression of these downstream genes. E75A has been found to play a crucial role in maintaining the “status-quo” function of JH during larval development, whereas E75C seems to be primarily involved in pupal-adult transition. The broad gene has been found to be a pupal specifying gene and thus plays a key role in pupal developmental program. The modulation of expression of these ecdysteroid induced transcription factors by JH indicates that JH plays a critical role in directing ecdysteroid signaling, although the modulation effect depends on the developmental status of the insect.

Key words: Insects, Molting, Metamorphosis, Juvenile Hormone, Ecdysteroid, E75, Broad

INTRODUCTION

The life of an insect is passed through a series of developmental events before it reaches the stage of reproductive adulthood. Holometabolous insects, such as flies and moths increase their body size through a series of larval molts. The transition from larval to pupal and adult forms is known as metamorphosis (literally a “change in form”) which is effected by the utilization of imaginal discs for the formation of completely new structures such as adult eyes, wings and genitalia and by the reprogramming of general body epidermis (Riddiford, 1980a). Depending on the degree of divergence between immature and mature forms, three categories of metamorphosis are seen in insects: ametaboly, hemimetaboly and holometaboly. Ametaboly is the most primitive pattern of development and is found in wingless insects of apterygote orders such as Thysanura and Archeognatha. In insect species showing ametaboly there is very little differences between immature and adult forms except for relative increase of body size and the presence of genitalia. Both the immature and adult occupy the same habitat and sexual maturity is obtained through series of molts. In hemimetabolous insects such as Rhodnius, grasshoppers, cockroaches the adults develop directly from nymph, and adult structures such as wing and genitalia are formed at the end of nymphal life. Like ametabolous insects, they also generally occupy similar niches. In holometabolous insect there is a radical change in the form and ecological habits between immatures and adults. In them the adult development passes through larval and pupal stages. This review focuses on the molecular basis of hormonal control of molting and metamorphosis in holometabolous insects.

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I. Role of Hormones in Molting and Metamorphosis

Insect molting requires periodic shedding of old cuticle and synthesis of new flexible cuticle that allows proper growth of body and then turns into rigid exoskeleton. Molting accompanied with a change in body form is known as metamorphosis. Based on works done by many researchers especially on *Drosophila*, *Bombyx mori* and *Manduca sexta* (Riddiford, 1980a, b, 1985, 1993, 1994, 1996, 2008; Sakurai, 1984; Sakurai *et al.*, 1998; Gilbert *et al.*, 2000; Hiruma, 2003; Riddiford *et al.*, 2003; Koyama *et al.*, 2004; Dubrovsky, 2005; Truman, 2005; Berger and Dubrovsky, 2005; Muramatsu *et al.*, 2008) the following conclusion can be drawn on hormonal control of insect molting and metamorphosis. The molting is characterized by a series of behavioural changes which can be observed before each molting process. It starts with the apolysis (retraction of epidermal cells from the inner surface of the old endocuticle) and ends with eclosion or ecdysis (the shedding of old cuticle). Eclosion is a term used for the emergence of adult from its pupal cuticle. Ecdlosion hormone secreted from neurosecretory cells (NSCs) of the brain controls the process of ecdysis/ecdlosion. Juvenile hormone (JH) and ecdysteroids (a generic term used in this review refer to ecdysone and 20-hydroxyecdysone, 20E) control and coordinate the overall process of molting and metamorphosis. The molting process is induced with the secretion of prothoracicotropic hormone (PTTH) by the NSCs of the brain in response to neural, hormonal and environmental cues. PTTH stimulates prothoracic gland (PTG) for the synthesis and release of ecdysone, which gets converted to 20-hydroxyedysone (20-HE) in the peripheral tissues such as fat body. The steroid hormone, 20-HE is responsible for the molting process but its action can be modulated by JH, which is being synthesized and secreted by corporal allata. Ecdysteroid in presence of JH causes larval molting but in its absence it causes larval-pupal or pupal-adult metamorphosis. Thus ecdysteroid initiates the molting process whereas JH determines the nature of the molt. The presence of JH has long been known to prevent metamorphosis of insects but in the present discussion we will see that there always exists a critical period for the presence or absence of JH which dictates larval–larval molting or metamorphosis. This critical period is very important and usually comes much before the actual molting process and is also sometimes referred to as commitment period or JH sensitive period. Williams (1952) coined the term “status-quo” for JH based on the fact that the application of JH containing extract at a developmentally sensitive period to pupa can induce a second pupal cuticle. Thus if JH is present at JH sensitive period which also coincides with the beginning of rise of ecdysteroid, it maintains the current developmental status but if JH is absent during that period there will be a change in a developmental status. The balance between JH and ecdysteroids thus defines the outcome of each developmental transition.

**Larval-larval molt**

A rising titer of ecdysteroid in presence of JH ensures larval-larval molt in insect species such as *Manduca sexta* and *Bombyx mori*, and the critical period for the presence of JH is at the time of initiation of ecdysteroid rise (Sakurai, 1983; Riddiford, 1985). In *Bombyx* this critical period can be identified externally by observing the spiracle apolysis, the first visible sign of larval molting (Kiguchi and Agui, 1981). The presence of high titer of JH before spiracle apolysis is critical for larval molt to happen, whereas after spiracle apolysis this hormone is irrelevant for the character of larval molt, although it still can affect the pigmentation of newly formed larval cuticle (Kiguchi, 1983). The rising ecdysteroid titer is needed for the actual molting process to happen. The ecdysteroid titer which starts rising during spiracle apolysis reaches to its maximum at the end of general apolysis and thereafter maintains at this level for some time to initiate all the events during the deposition of new cuticle (Kiguchi and Agui,
In case of fourth instar larva of *Manduca* the removal of corpora allata, the source of JH before the critical period for the presence of JH at the time of ecdysteroid rise, causes the formation of precocious pupa instead of fifth instar larva. The role of JH in larval molt is further evident from the fact that the application of JH to allatectomized larva prevents the formation of precocious pupa and restores the larval molt (Hiruma, 2003).

**Larval-pupal commitment period and pupal molt**

The endocrine events which initiate larval–pupal metamorphosis are completely different from larval-larval molting. During the final larval instar, the JH titer declines due to a cessation of synthesis by corpora allata and increased degradation primarily by JH esterase in the hemolymph and target tissues (Tobe and Stay 1985). In *Bombyx*, a low level of ecdysteroid is responsible for the initiation of complete inactivation of corpora allata and thus ensures larval–pupal metamorphosis as JH titers decreases (Gu *et al.*, 1996; Kaneko *et al.*, 2011). The time of release of PTTH is critical for pupation to occur. When the larva attains critical weight and JH level declines to undetectable level, the PTTH from NSCs of brain is released which in turn activates PTG for the synthesis and release of ecdysone (Nijhout 1981, Sakurai, 1984; Rountree and Bollenbacher, 1986). In *Manduca* there occurs a small peak of ecdysone, also known as commitment peak in absence of JH, which is the critical period for larval-pupal commitment of the epidermis and is responsible for switching of larval-pupal developmental program (Riddiford, 1976). The application of large amount of JH before this critical period causes the formation of sixth instar larva instead of pupa, whereas its application after the critical period has no effect on pupation (Hiruma, 2003). In *Drosophila*, the JH treatment produce pupal-adult intermediates by preventing the adult differentiation of central nervous system, muscles and abdominal epidermis but it does not affect larval molting and pupation (Hiruma, 2003; Wilson, 2004).

In *Bombyx*, the JH treatment before the gated release of PTTH can only prolong the larval duration but is not causing the formation of sixth instar larva as found in *Manduca*. In *Bombyx* the last instar larva is destined to undergo pupal metamorphosis at the time of the fourth larval ecdysis only (Sakurai, 1984). Therefore the mechanism of pupal commitment in *Bombyx* might be different from that of *Manduca*. Muramatsu *et al.* (2008) showed that in *Bombyx* the competence phase of pupal commitment of the epidermis starts 6h before fourth larval ecdysis and continues till day 2 of 5th instar and the commitment phase occurs from day 3 to day 6 of last instar larva. During the competence phase the pupal commitment of epidermis can be induced by 20-HE *in vitro* and inhibited by JH (Muramatsu *et al.*, 2008). The commitment phase induces the initiation of larval-pupal metamorphosis which is characterized by the cessation of feeding followed by emptying of gut content (known as ‘gut purge”) and wandering behaviour (Kiguchi, 1983; Riddiford, 1985). In *Bombyx* “gut purge” occurs after a feeding period of 6-7 days in final larval instar and 4-5 days afterwards pupation ensues. The second PTTH release occurs around the time of gut purge and thus the ecdysteroid titer also rises rapidly in response to PTTH stimulation on PTG and reaches maximum just before larva finishes cocoon spinning. In *Manduca* two days after this commitment peak and after the start of wandering behaviour, there is an ecdysteroid surge along with that of JH (Kiguchi and Riddiford, 1978). This prepupal JH is necessary to prevent precocious adult differentiation of the imaginal discs during the pupal molt and the exceptions are *Bombyx* and *Galleria* in which allatectomized larvae lacking JH, form apparently normal pupae (Riddiford, 1995).

**Pupal-adult molt**

The critical period for adult development in *Bombyx* is about 12-18h after pupal ecdysis when ecdysone is secreted from PTG. In *Manduca* during this critical period a small amount of
ecdysonic acid level is present in absence of JH and this rising ecdysteroid causes switching to adult commitment followed by adult differentiation (Riddiford, 1985). JH treatment during this critical period prevents adult development and causes the formation of second pupal cuticle. The surge of ecdysteroid after 5-10 days of pupation is responsible for the formation of adult structures (Hiruma, 2003).

II. Molecular Basis of Hormone Action

The molecular mechanisms by which ecdysteroids act and coordinate many cellular events have been extensively studied in Drosophila. The first classical model showing the molecular mechanism of ecdysteroid action was proposed by Ashburner et al. (1974) on puffing activity in Drosophila salivary gland chromosome. According to this model, the ecdysone–receptor complex activates the expression of early genes, which in turn initiates the expression of late genes. They also act to repress their own expression. Many of the later studies have confirmed this model. The studies over the past two decades have provided a detailed understanding of the molecular mechanisms by which nuclear receptors convert a hormonal signal into a transcriptional response.

20-HE exerts its effect through binding with its nuclear receptor, which is a heterodimer of ecdysone receptor (EcR) (Koelle et al., 1991) and ultraspireacle (USP) (Yao et al., 1992) proteins. The nuclear receptors are one family of transcription factors which show conserved DNA-binding domain (DBD) and lignand-binding domain (LBD). The Drosophila melanogaster genome project revealed 18 nuclear receptors with DBD and LBD (Fahrbach et al., 2012). Many of the early genes involved in ecdysteroid signaling pathway are the members of nuclear-receptor superfamily.

20-HE-EcR-USP complex coordinates downstream gene expression and JH modulates the action of ecdysteroid on molting and metamorphosis through these downstream genes. In addition to EcR, other nuclear receptor genes such as DHR3, DHR4, DHR39, E75, E78, and ftz transcription factor 1 are transcriptionally regulated by 20-HE and show marked changes in mRNA levels in synchrony with ecdysteroid pulses during development (Riddiford et al., 1999; King-Jones and Thummel, 2005; Hiruma and Riddiford, 2010).

The transcription factor broad which appears at metamorphosis in both Drosophila and Manduca shares the BTB protein interaction domain at the N-terminus with a group of transcription factors that are known to be involved in the alteration of chromatin structure (Albagli et al., 1995). The protein products of these early genes directly or indirectly perform distinct metamorphic processes such as cell death of obsolete larval tissues, cell proliferation and differentiation of imaginal tissues, and pupal cuticle formation (Thummel, 2002). Hiruma et al. (1999) showed that JH modulates the ecdysteroid regulated expression of EcR and USP genes in an isoform specific manner. It also modifies the expression of early genes such as E75 and broad depending on the developmental sensitive period. This review will highlights some of our understanding on the role of E75 and broad, the most studied transcription factors in insect molting and metamorphosis.

Role of E75

E75 is one of the ecdysteroid induced early genes, which has been identified in several insect species including Drosophila, Manduca and Bombyx (Feigl et al., 1989; Segraves and Hogness, 1990; Segraves and Woldin, 1993; Zhou et al., 1998a; Swevers et al., 2002; Dubrovskaya et al., 2004 a, b; Keshan et al., 2006). E75 has four different isoforms, each with a distinctive N-terminal A/B domain and developmental profile. A loss-of-function mutation of E75 in Drosophila, which eliminates all the isoforms, causes lethality during late embryonic stage or during first larval instar (Bialecki et al., 2002). The mRNA expression of MsE75A was found in abdominal epidermis during both larval and pupal molts and at pupal commitment period and is transiently induced by 20-HE (Zhou et al., 2003).
et al., 1998a). MsE75D is expressed at the time of pupal commitment, at the end of larval and pupal molt and at the beginning of deposition of cuticle during all the molts (Keshan et al., 2006). A small increase of ecdysteroid on day 3 of 5th larval instar in Manduca causes the epidermal cells to become pupally committed, but when JH is present at or before this critical time, it prevents the pupal commitment (Riddiford, 1976, 1978). An in vitro study showed that the presence of JH at this critical period causes a rise in 20-HE-induced increase of MsE75A mRNA, whereas a suppression of MsE75D mRNA (Zhou et al., 1998a; Keshan et al., 2006). These studies thus indicate that high E75A may be important for maintenance of the larval state and that the appearance of E75D mRNA in response to 20-HE is a part of the pupal commitment process. E75C expression was primarily found during adult development in Manduca (Keshan et al., 2006) and during prepupal period and adult development in Drosophila (Dubrovsky et al., 2004b). MsE75C mRNA was found to be suppressed by JH during second pupal development indicating its role in adult differentiation program (Keshan et al., 2006). A loss-of-function mutation in E75C causes defects in adult development in Drosophila and most die as pharate adults (Bialecki et al., 2002). Thus E75C seems to be primarily involved in the pupal-adult transition.

The alterations in E75 expression by JH shows that JH modulates ecdysteroid action by acting on downstream genes in ecdysteroid signaling pathway but its action depends on the developmental status of the insect. Thus the study on E75 shows that it is a common element in both the JH and ecdysone signaling pathway (Dubrovskaya et al., 2004 a, b).

Role of broad

The broad gene is one of the 20-HE-induced transcription factors that play a key role in the pupal development program as its expression in epidermis is found only from the time of pupal commitment through the time of pupation in both Drosophila and Manduca (Zhou and Riddiford, 2002). These genes are not generally expressed during larval or adult molt. In Manduca the first expression of broad in epidermis occurs during the small peak of ecdysone in absence of JH to cause pupal commitment at the onset of metamorphosis, and then persist during the prepupal period (Zhou et al., 1998b; Zhou and Riddiford, 2001). Application of JH before this pupal commitment period prevents the appearance of broad and the larval-pupal transformation, whereas its treatment before the onset of adult molt causes the re-expression of broad and the formation of second pupal cuticle in both Drosophila and Manduca. Ectopic expression of one of the isoforms of broad either during a larval or an adult molt of Drosophila causes the activation of pupal cuticle genes and suppression of larval or adult cuticle genes (Zhou and Riddiford, 2002; Bayer et al., 2003). Thus, during the critical period of adult commitment, ecdysteroid in absence of JH must switch off broad expression so that normal adult differentiation can occur. As broad can both activate pupal genes and suppress adult specific genes, its re-expression during second pupal development by JH treatment has shown its role as a mediator of “status-quo” action of JH. Drosophila mutants that lack broad develops normally but only till final larval instar (3rd instar) but cannot undergo larval-pupal transformation and died before pupation (Kiss et al., 1976, 1988). In Bombyx 20-HE has been found to cause the induction of broad mRNA expression whereas JH suppresses this induced expression in tissues of last instar larva (Reza et al., 2004). The broad expression in Bombyx was found during larval-pupal transformation but not during the penultimate instar (Reza et al., 2004). Its expression increases during the spinning ecdysteroid peak when pupal commitment occurs in epidermis (Jiro et al., 2004). The expression of broad thus can be used as a molecular marker for pupal commitment process in Bombyx as it closely follows the pupal commitment process (Muramatsu et al., 2008).
RNAi knockdown experiments in *Bombyx* confirms the findings that *broad* is important in metamorphosis as RNAi disrupts the differentiation of adult compound eyes, legs, and wings besides preventing the programmed cell death of larval silk glands (Uhlirova *et al*., 2003). All these findings explain the metamorphosis-specific expression of *broad* during pupal development and strengthen the fact that it plays a critical role in the larval-pupal metamorphosis regulated by both ecdysteroid and JH.

The modulation of various ecdysteroid induced nuclear receptors by JH shows that JH plays a crucial role in directing ecdysteroid signaling although it is very much dependent on the physiological and developmental status of an insect. In fact, Juvenile hormone is not interfering with the molting response of the ecdysteroids but it prevents the ecdysteroid-induced expression of downstream genes necessary for metamorphosis. Further studies will give us more insight on the role of JH and ecdysteroid on the regulation of the transcription factors and thereby on molting and metamorphosis.

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