Ecdysone receptor B1 in *Bombyx mori* L. (*Lepidoptera:Bombycidae*) prothoracic gland under various organ culture conditions

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Abstract

During the early last larval instar, hormone secretory activity of prothoracic glands in *Bombyx mori* L. is undetectable despite the high prothoracicotropic hormone (PTTH) level in hemolymph. Early reports indicated that the inhibition of gland activity is caused by 20-hydroxyecdysteroid (20E) peak on day 2 of the fourth larval instar and juvenile hormone (JH) only resumes this inactive period during the early days of 5th larval instar. Following these reports, prothoracic glands also become a target of its hormone, 20E. Actions of 20E are mediated via heterodimeric ecdysone receptor complex which consists of ecdysone receptor (EcR) and its partner molecule, ultraspiracle (USP). This study was designed to determine the expression pattern of ecdysone receptor B1 (EcR-B1) in the prothoracic glands of *B. mori* during last larval instar and early pupal stage. Organ culture experiments were carried out to clarify the effects of juvenile hormone and 20-hydroxyecdysone on the EcR-B1 expression. Immunocytochemical studies revealed the presence of EcR-B1 in prothoracic gland cells. A rise of EcR-B1 immoreactivity was detected on day 3 of 5th instar in which glands recover the secretion activity. Juvenile hormone administration to culture medium maintained EcR-B1 immunoreactivity especially on day 6 and day 9 when compared with control but in contrast to this result, an ecdysone application to culture medium weakened the immunoreactivity. These results suggested that negative feedback effect of ecdysone on secretory activity of the glands may be exerted by means of ecdysone receptor and it may also involve consistent and regular 20E secretion during the fifth instar.

Keywords: *Bombyx mori*, ecdysone, ecdysone receptor B1, fenoxycarb, juvenile hormone, prothoracic gland, silkworm
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Farklı organ kültürü koşullarında *Bombyx mori* (*Lepidoptera:Bombycidae*) protorasik bezinde ekdizon reseptör B1

Özet

Son larval evrenin ilk günleri boyunca *Bombyx mori L*. protorasik bezindeki salgı aktivitesi hemolenfteki yüksek protorasikotropik hormon miktarına rağmen belirlenemez düzeydedir. Önceki çalışmalar, bez aktivitesindeki bu baskılanmanın dördüncü larval evrenin 2 günündeki 20-hidroksiekdizon (20E) pikinden kaynaklandığını ve juvenil hormonun bu inaktif periyodu 5.larval evrenin ilk günleri boyunca sürdürdüğünü belirtmektedir. Bu çalışmaların neticesinde, protorasik bez aynı zamanda kendi hormonu olan, 20E'nun, bir hedefi haline gelmektedir. 20E'nun etkileri ekdizon reseptörü (EcR) ve onun partner molekülü, ultrasipirakıl (USP)'ı içeren heterodimerik ekdizon reseptör kompleksi aracılığı ile gerçekleşir. Bu çalışma son larval evre ve erken pupal dönem boyunca *B. mori* protorasik bezinde ekdizon reseptör B1 (EcR-B1)'in ekspresyon düzeyini belirlemek için tasarlanmıştır. Organ kültürü denemeleri juvenil hormonun ve 20-hidroksiekdizonun EcR-B1 ekspresyonu üzerindeki etkilerini açıklığa kavuşturmak için gerçekleştirilmiştir. Immunositokimyasal çalışmalar EcR-B1'in protorasik bez hücrelerinde var olduğunu göstermiştir. Bezin salgı aktivitesini yeniden kazandığı 5.larval evrenin 3. gününde EcR-B1 immünreaksiyonunda bir artış belirlenmiştir. Kültür ortamına juvenil hormon uygulanması

kontrol grubuyla karşılaştırıldığında özellikle 6. ve 9. günlerde EcR-B1 immünreaksiyonu devam ettirmiştir, ancak bu sonuca zıt olarak, kültür ortamına ekdizon uygulanması immunreaksiyonun zayıflamasına neden olmuştur. Bu sonuçlar bezin salgı aktivitesi üzerinde ekdizonun negatif geri bildirim etkisini ekdizon reseptörü aracılığı ile gerçekleştirebileceğini ve aynı zamanda 5. larval evre boyunca sürekli ve düzenli 20E salınımında da rol oyanayabileceğini göstermektedir.

Anahtar Kelimeler: *Bombyx mori,* ekdizon, ekdizon reseptör B1, fenoksikarb, ipekböceği, juvenil hormon, protorasik bez.

Introduction

Insect molting and metamorphosis are mainly controlled by a steroid, 20-hydroxyecdysone (20E) which originates from the prothoracic glands (PG) and sesquiterpenoid juvenile hormone (JH) which is secreted from the Corpora Allata. The regulation of PG activity in *Bombyx mori* L. has been investigated in various studies (Takaki and Sakurai, 2003; Mizoguchi and Kataoka, 2005). Prothoracicotropic hormone (PTTH) enhances the PG secretory activity but this is not the only way for regulating the secretory activity of the glands. Hemolymph PTTH and 20E concentrations do not show an expected correlation during first three days of 5^{th} instar. Despite high PTTH level, PG does not respond to the PTTH signal and 20E concentration remains at an undetectable level. Studies during the early days of fifth instar indicated that some other factors like JH and 20E are also involved in this inhibition (Sakurai et., al., 1989; Takaki and Sakurai, 2003), but their molecular mechanism of action is still unclear. Inactivity of PG in the early 5^{th} instar is carried out by feedback inhibition by 20E occurring in the late 4^{th} instar (Takaki and Sakurai, 2003). Negative feedback mechanism of 20E on PG has been reported in *Manduca sexta* L. during fifth instar, prepupal and pupal period (Sakurai and Williams, 1989; Song and Gilbert, 1998). Mizoguchi and Kataoka, (2005) reported that inactivity of the gland is not the result from hemolymph JH level but JH may act only preventing the reactivation of the gland. According to those reports, PG is also the target organ of 20E.

20E exerts its effects via a heterodimeric nuclear receptor complex, consisting of the ecdysone receptor (EcR) and Ultraspiracle (USP). Three isoforms of ecdysone receptor EcR-A, EcR-B1 and EcR-B2 have been characterized in *Drosophila* (Meigen) (Koelle et. al., 1991; Talbot et. al., 1993), and two isoforms in *Manduca* (Jindra et. al., 1997) and *Bombyx* (Swevers et. al., 1996). EcR expression was reported in various tissues such as the anterior silk gland (Kamimura and Kiuchi, 1997; Goncu and Parlak, 2009, midgut (Kamimura and Kiuchi, 1997) and fat body (Kamimura and Kiuchi, 1997) of *Bombyx mori*, but no information is available about EcR expression in PG of *B. mori*. Song et. al. (1997) reported that developmental profiles of EcR deduced from immunocytochemical analysis showed two peaks of EcR on day 3 and day 7 of fifth instar in *M. sexta.*

In the present study, we cultured *B. mori* PGs under various conditions and demonstrated stage specific expression of EcR-B1. According to the EcR receptor status in PG, our results suggest that a high 20E concentration inhibited EcR-B1 expression and juvenile hormone analogue fenoxycarb stabilized protein in the PG. EcR-B1 may be involved in the regulation of the PG secretory activity by 20E and JH during fifth instar.

Materials and methods

Experimental animals

Bombyx mori larvae were reared on fresh mulberry leaves at 25±1°C and high humidity (>65%). Relatively synchronous populations of animals were selected by routine staging on day 0 of 5th instar and at pupation. In our rearing regimen, animals started spinning on day 7 of fifth instar, and pupation occurred on day 10.

Hormones and chemicals

20-Hydroxyecdysone was obtained from Rohto Pharmaceutical Co.Ltd. Osaka Japan. It was dissolved in water and stored at -20C until use (0.5µg/ml). Fenoxycarb (Ethyl[2-(p-phenoxy-phenoxy)ethyl] carbamate:$C_{17}H_{19}O_4N$ of an analytical grade (99% purity) was obtained from Riedel (31343). A stock solution of fenoxycarb was prepared in dimethylsulfoxide (DMSO). Working solutions were prepared with different concentrations as 100ng/ml and 10ng/ml from the stock solution.

In vitro prothoracic gland assay

Prothoracic glands of day 0, 3, 6, 9 and 10 of fifth instar larvae and P0 (at pupation) and P24 (24 hr after pupation) pupae were cultured in Grace's insect medium for 24 hr under various endocrine conditions. These days were chosen according to the hemolymph 20E levels during 5th instar (Sakurai, et., al. 1998). After day 9 of 5^{th} instar, experiments were performed every 24hr to observe the changes in the gland during prepupal period and just after pupation. Larvae were anaesthetized on ice and PGs were dissected from each larva in sterile insect physiological saline (0.85% NaCl). Two pairs of glands were used for experiments. Under saline, the thorax of larvae was open at ventral side and the PGs were cut off with scissors. They were rinsed with saline and cultivated in 100µl of Grace's insect medium with various contents in wells of a 96-well cell culture plate. The plate was maintained at 25°C for 24 h.

Immunocytochemistry

EcR-B1 protein expression was examined by immunocytochemistry. The cultured prothoracic glands were fixed for 0.5h in freshly prepared 3.7% formaldehyde in phosphate buffered saline (150mM NaCl, 2 mM NaH_2PO_4, 7 mM Na_2HPO_4; PBS), then incubated with 6B7 EcR-B1 specific monoclonal antibody (Developmental studies of Hybridoma Bank, DSHB) in PBS (1:700) overnight at 4 °C followed by an incubation for 2hrs with 1:200 horseradish-peroxidase conjugate anti-mouse secondary antibody (Sigma) (Jindra et. al., 1996). Visualization was performed with diaminobenzidine (DAB). The samples were examined under an Olympus BX-51 microscope and photographed with an Olympus digital camera.

Image analysis and statistical analysis

Image analyses were carried out by using image J analysis software from the National Institutes of Health, U.S.A. (http://rsb.info.nih.gov/nih-image/). The intensity of EcR-B1 immunoreaction was measured by the comparison of the area fraction value in the 8 bit gray scale. The values of area fraction for EcR-B1 immunosignal are presented as a mean ± S.D. The significance of differences between the intensity of EcR-B1 immunoreaction in control and experiment groups of prothoracic glands were calculated by using one way ANOVA. $p \leq 0,05$.

Results

Control group

The control group PG cells showed moderate immunoreactivity on day 0 (scored 0.9) (Fig. 1a). Immunosignal became stronger on day 3 and the immunostaining was more prominent in the cytoplasm (Fig. 1b). As the larval stage proceeds, the expression of EcR-B1 in the PG gradually declined. Moderate signal was seen on day 6 (Fig. 1c) and day 9 (Fig. 1d) glands after 24h culture. Labeling was determined in the cytoplasm. Day 10 glands had weak intensity of EcR-B1 immunoreactivity (Fig. 1e). At pupation (P0), cell boundaries and intercellular spaces were clearly distinguishable. This observation indicated that cells detached from each other and it may be the first sign of programmed cell death of PG (Fig. 1f). Immunolabelling was not detected after 24hr of pupation (Fig. 1g). No immunostaining was seen when the incubation was carried out with omission of the primary antibody (Fig. 1h).

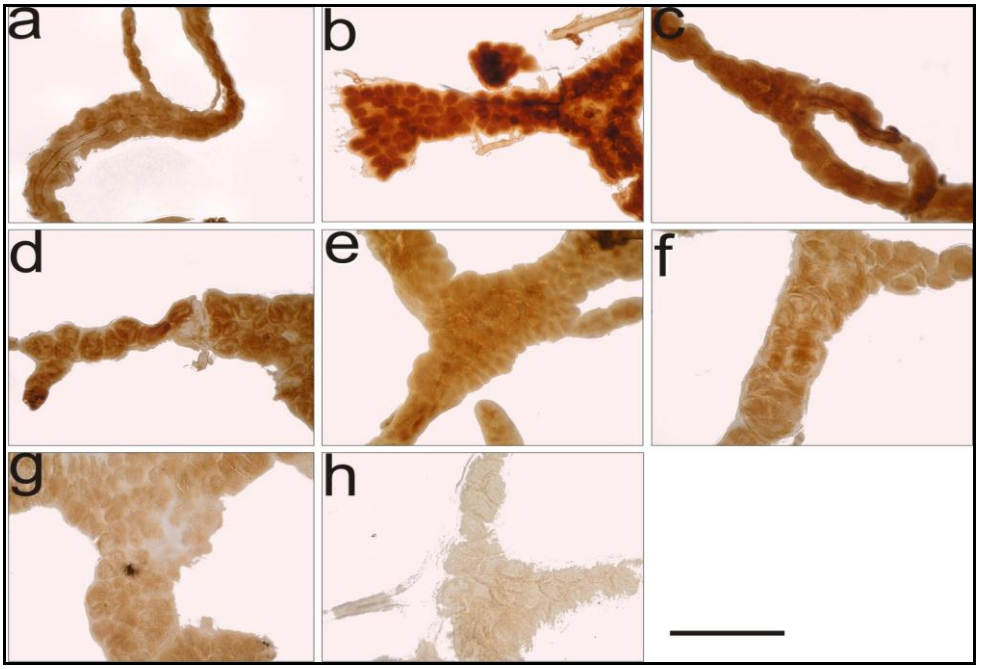

Figure 1. Control groups, prothoracic glands were cultured in Grace's insect medium for 24 hr and EcR-B1 protein was detected by using immunocytochemistry. **(a)** Day 0; **(b)** day 3 glands showed strong immunolabelling in cytoplasm; **(c)** dissected PGs on day 6 showed moderate immunoreaction after 24 hr culture; labelling intensity become weaker after **(d)** day 9 and **(e)** day 10; **(f)** immunoreactivity were determined weak just before pupation; **(g)** 24 hr pupae PGs had no DAB signal. **(h)** Negative control, cultured prothoracic silk gland on day 3 showed no immunolabelling. Scale bar 325 μm for a, b; 650 μm for c, d, e, f, g and h.

Fenoxycarb treatment groups

When PGs were cultured with fenoxycarb (10ng/ml and 100ng/ml), they showed similar immunolabelling independent of concentrations (See Fig. 2 and 3). Moderate immunoreactivity was determined on day 0 (Figs 2a; 3a) and almost similar immunoreactivity was observed in day 3 treated glands (Figs 2b; 3b). Day 6 treatment did not affect EcR-B1 expression if compared with control, and the immunoreactivity was found almost similar to day 3 treatment group (Figs 2c; 3c). EcR-B1 expression persisted in day 6, day 9 and day 10 treatment groups (Figs 2c; 2d; 2e – 3c; 3d; 3e). After pupation, morphological changes did not occur as clearly as in the control. The sharp decline in immunoreactivity was determined in P0 (Figs 2f; 3f) and p24 (Figs 2g; 3g) treatment groups. We did not find significant differences in the mean area fraction values for EcR-B1 when compared with controls (Fig 7).

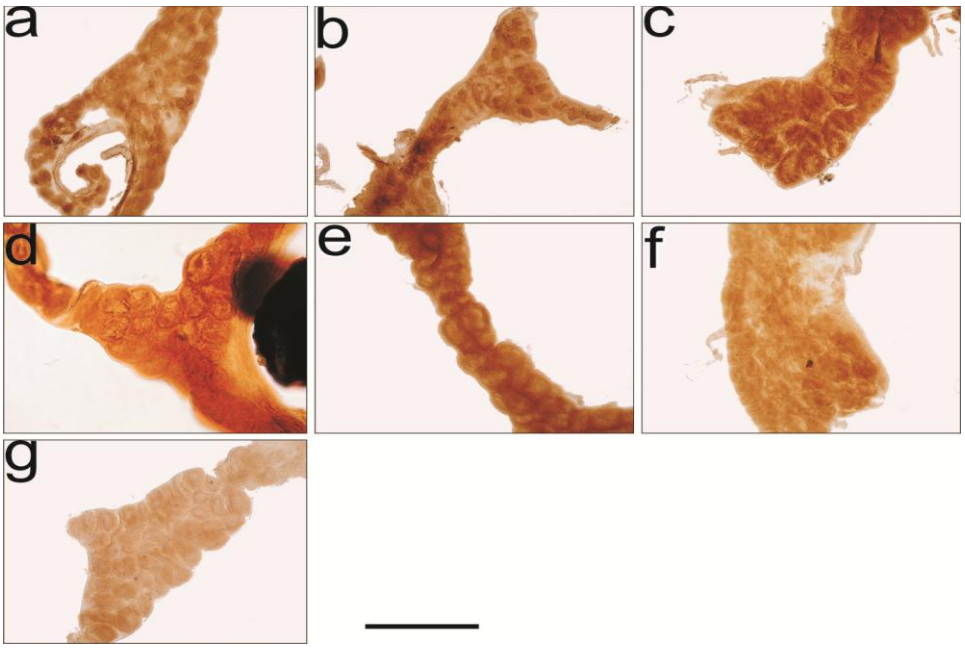

Figure 2. Prothoracic glands were cultured with 1ng/100µl juvenile hormone analogue fenoxycarb for 24 hr and EcR B1 protein was detected. **(a)** Immunosignal was similar with control on day 0; **(b)** moderate labelling intensity was determined on day 3; persistence immunoreactivity were observed on **(c)** day 6, **(d)** day 9 and **(e)** day 10. Immunolabelling declined after larval-pupal ecdysis **(f)** at pupation, and **(g)** 24 hr pupae. ecdysone application to culture medium reduced positive cell number and immunoreaction persisted in nucleus: **(e)** ecdysone + 1 ng fenoxycarb and **(f)** ecdysone + 10 ng fenoxycarb treated groups had similar immunoreactivity with control group. Scale bar 325 µm for a, b; 650 µm for c, d, e, f and g.

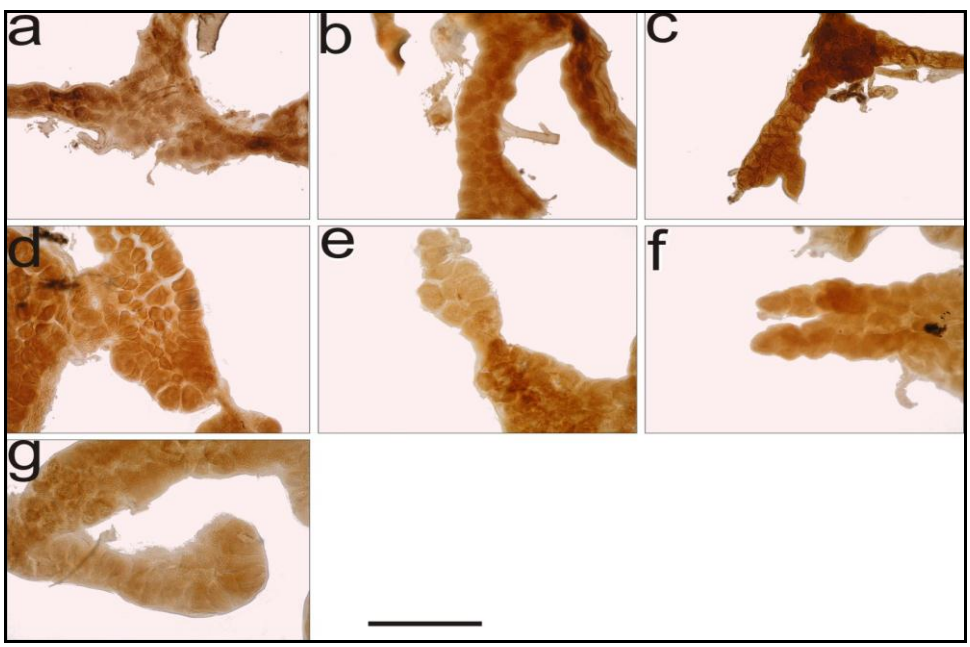

Figure 3. Prothoracic glands were cultured with 10ng/100µl juvenile hormone analogue fenoxycarb for 24 hr and EcR B1 protein was detected. Almost similar results to those with 1ng/100µl fenoxycarb application group were obtained. **(a)** day 0, **(b)** day 3, **(c)** day 6, **(d)** day 9, **(e)** day 10, **(f)** at pupation, **(g)** 24hr pupae. Scale bar 325 µm for a, b, c; 650 µm for d, e, f, g.

Ecdysone treatment groups

20E (0.5µg/ml) was applied to culture medium and PGs were cultured for 24 hr. The PG cells became enlarged and immunoreactivity was weaker than control in day 0 treatment group (Fig 4a). Ecdysone application to gland culture on day 3 reduced the positive cell numbers and immunosignaling was dominantly determined in the nucleus (Fig 4b). Immunosignaling was weakening in day 6 and day 9 treatment groups (Fig 4c; 4d). Almost similar results were obtained on and after day 10 (Figs 4e, 4f, 4g). The mean area fraction value for EcR-B1 was found significantly lower than the control (Fig 7).

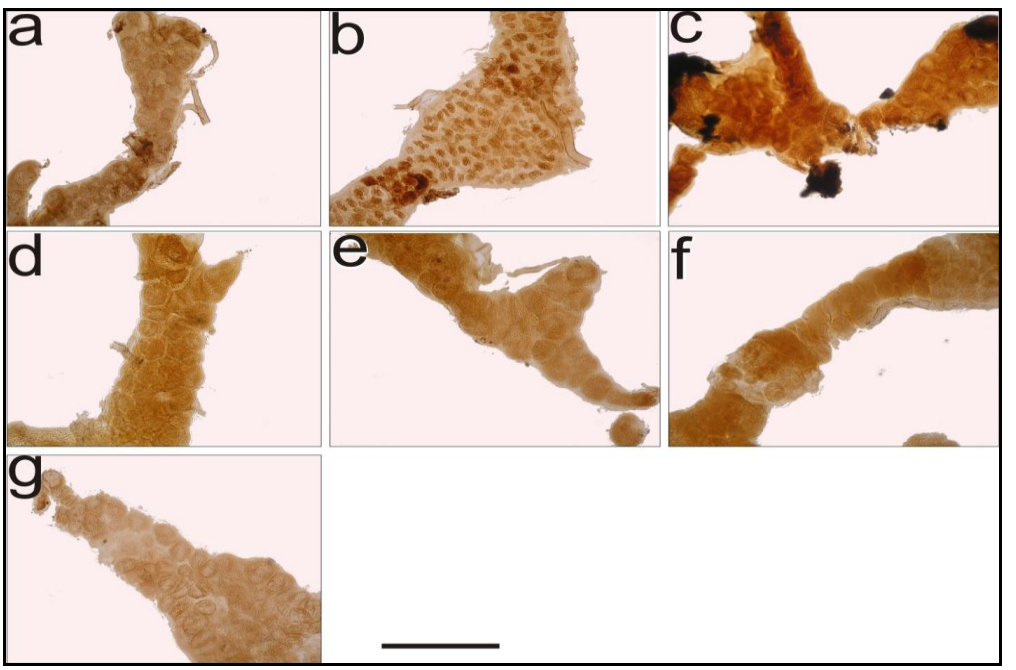

Figure 4. Prothoracic glands were cultured with 0.5 µg/ml ecdysone. **(a)** Immunoreactivity was weak when compared with the control on day 0; **(b)** moderate labelling intensity was determined in the nucleus; **(c)** immunolabelling increased on day 6 experiments when compared to day 3 but it probably related to in vivo conditions before dissection. DAB signal decreased day by day from **(d)** day 9 to **(g)** 24 hr pupae. Scale bar 325 µm for a, b; 650 µm for c, d, e, f and g.

Fenoxycarb + ecdysone treatment groups

Two different doses of fenoxycarb (10ng/ml and 100ng/ml), were applied with one dose of 20E (0.5µg/ml) to the glands culture medium. Both treatment groups exhibited a similar pattern of EcR-B1 distribution among PG cells during the experiments (Figs. 5 and 6). EcR-B1 expression was completely inhibited by the treatments in day 0 glands (Figs 5a; 6a). No immunoreactions were determined in these groups. Day 3 experiments showed similar immunolabelling with the control (Figs 5b; 6b), and labeling intensity was strong. Day 6 experiments showed that, fenoxycarb and ecdysone together relatively reduced the immunolabelling of gland cells (Figs 5c; 6c and Figs 3c; 4c). Moderate immunolabelling was determined in day 9 treatment groups (Figs 5d; 6d). On day 10, just before pupation, PG cells had weak intensity of EcR-B1 immunoreactivity (Figs 5e; 6e). We did not find a significant difference in the mean area fraction values for EcR-B1 when compared with the control (Fig 7). After larval-pupal ecdysis, cell detachments in prothoracic glands were obvious and labeling intensity decreased in early pupal stage (Figs 5f, 5g; 6f, 6g).

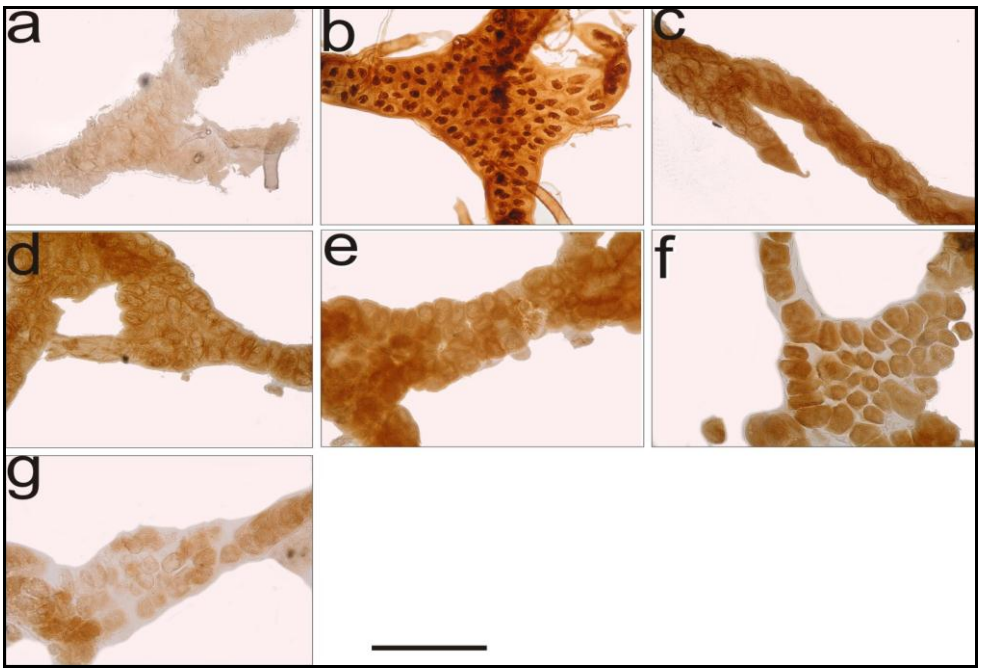

Figure 5. Prothoracic glands were cultured with 1 ng/100µl fenoxycarb and 0.5 µg/ml ecdysone. **(a)** Immunoreactivity was weak on day 0; **(b)** day 3 application showed similar immunolabelling with control; **(c)** after day 6, labelling intensity decreased: **(d)** day 9, **(e)** day 10, **(f)** at pupation, **(g)** 24hr pupae. Scale bar 325 µm for a, b; 650 µm for c, d, e, f and g.

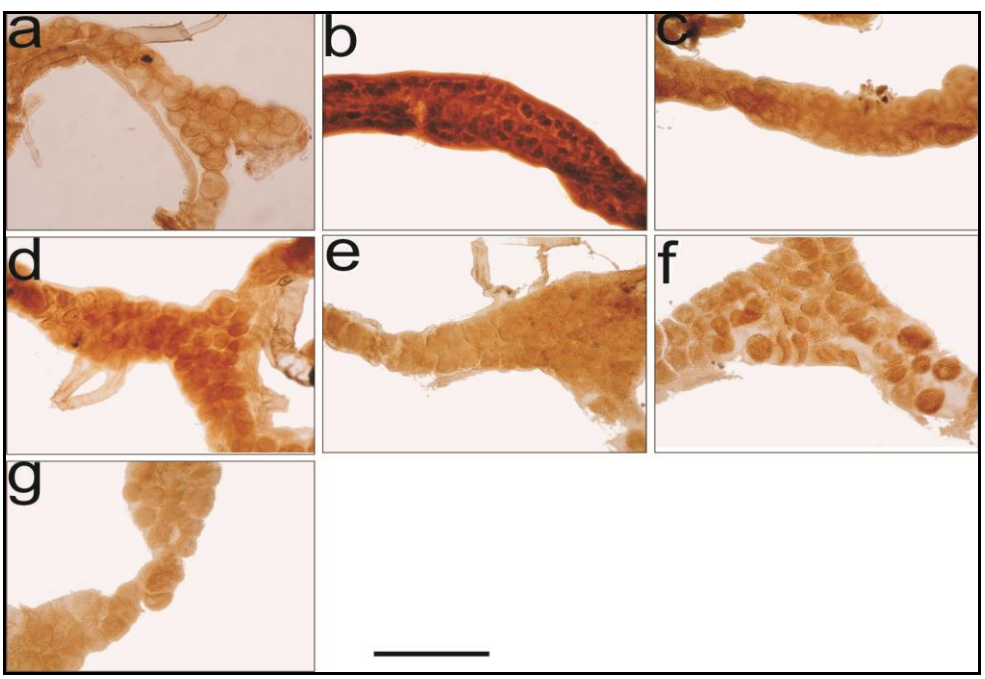

Figure 6. Prothoracic glands were cultured with 10 ng/100µl fenoxycarb and 0.5 µg/ml ecdysone. **(a)** EcR-B1 expression was inhibited on day 0; **(b)** the strongest labelling intensity was determined on day 3; **(c)** day 6 cultured glands showed moderate immunolabelling; **(d)** immunoreactivity declined after day 9 like in control: **(e)** day 10, **(f)** at pupation, **(g)** 24 hr pupae. Scale bar 325 µm for a, b, c; 650 µm for d, e, f, g.

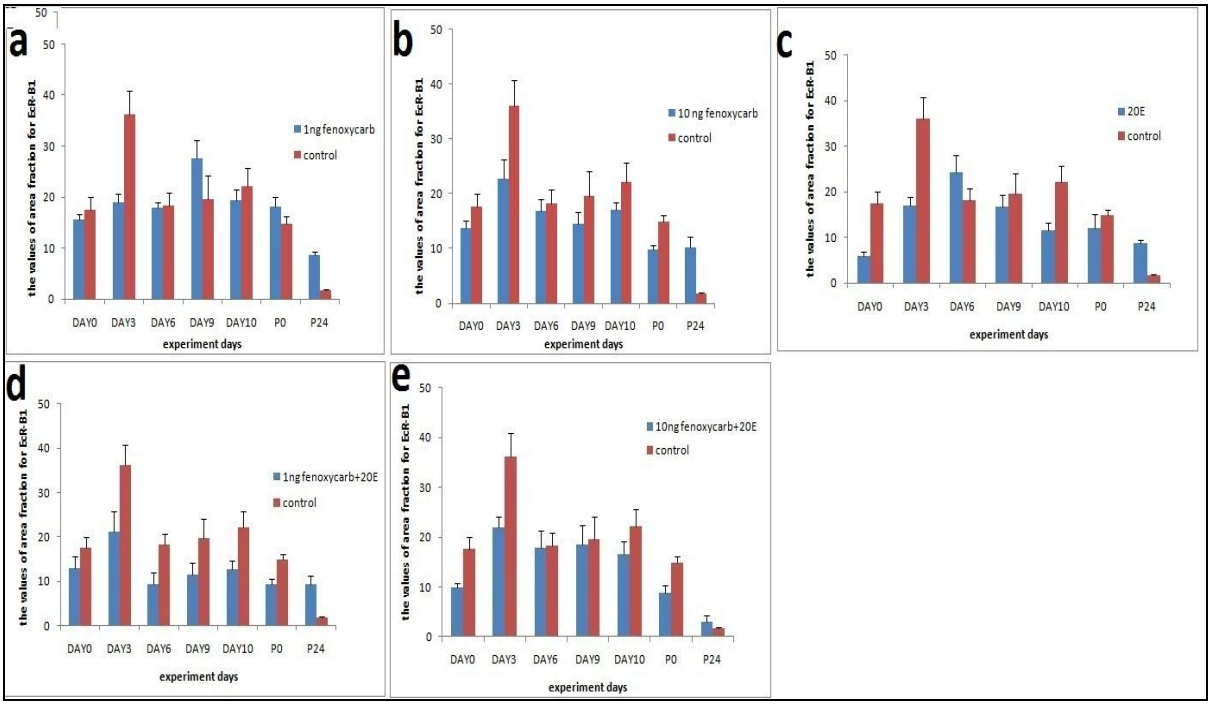

Figure 7. The values of area fraction for EcR-B1 were calculated by using Image J analysis software from the National Institutes of Health, US (http://rsb.info.nih.gov/nih-image/). **a)** control and 1 ng fenoxycarb treated groups, **b)** control and 10ng fenoxycarb treated groups, **c)** control and 20E treated groups, **d)** control and 1 ng fenoxycarb+20E treated groups, **e)** control and 10ng fenoxycarb+20E treated groups. Values are expressed as means±SD. Significant difference was found between 20E treatment groups and control groups by using one way ANOVA, $p<0.05$, $n=7$

Discussion

PG of *B. mori* can be divided into three part morphologically; anterior branches, main trunk and posterior branches. These branches generally consist of large cells and the main trunk is comparatively composed of small cells. Functional differences between these cells were not reported in early studies (Akai, 1998). Ecdysone release mechanism during 5th instar is different from other larval instars and this secretion pattern is important for larval-pupal metamorphosis. Despite the high PTTH level in the early 5th instar, PG does not show ecdysone secretion activity during those days, which indicates that inhibition of PG activity is not resulting from the low PTTH level (Mizoguchi et. al. 2001). Mizoguchi and Kataoka (2005) reported that repression of PG is related to high ecdysteroid concentrations on day 2 of 4th instar. Feedback mechanism prevents ecdysteroid secretion and JH resumes this inactivity period. According to these reports, PG is a target of ecdysone. The influence of ecdysone and juvenile hormone on PG can be studied with the nearest connection to a possible ecdysone related activity regulation. So far, there are only a few investigations concerning ecdysone receptors in PG. Ecdysone receptors were detected in isolated PGs from *M. sexta* and *Blattella germanica* L.(Song et. al. 1997). Song et al. (1997) suggested, if negative feedback is related to gland inactivity during early three days of fifth instar of *M. sexta*, EcR must act a role during this process. Beydon and Lafont (1983) reported that 20E is a negative regulator for ecdysone production in *Pieris brasscicae* pupae and 20E affects directly upon the PG.

Control group

Although high PTTH level in the hemolymph, secretory activity of the PGs was undetectable in the early stage of 5th instar. These results confirmed that PGs are insensitive to PTTH during these days. JH titer was found high in the hemolymph but gradually decreased until day 3 (Niimi and Sakurai, 1997). PG cells showed moderate immunoreactivity on day 0 (Fig 1a). The glands gradually become active and sensitive to PTTH on day 3. When day 3 control group was considered, all PGs have EcR-B1 protein and the stainability was found to be stronger than other experiment days. This result may arise from exposure of the gland to in vivo endocrine conditions before the experiment. Early studies indicated that PGs from early 5th instar contain cholesterol for ecdysone biosynthesis as 30 ng/gland. This level is too high when the total ecdysteroid amount released from day 0 glands during four days in vitro culture is considered. Those glands also continue ecdysone secretion during one week without supplement any substrate for secretion. These reports suggest that day 0 PG cells have a large ecdysone precursor pool for regular and continuous 20E secretion after day 3 of 5th instar unlike other larval instars (Sakurai et. al. 1986). This 20E release mechanism may be regulated by JH and feedback mechanism of ecdysone. EcR-B1 expression in day 3 experiment was found higher than other experiment days. This high EcR-B1 presence may be related to gland activity and be involved in the regulation of continuous ecdysone secretion from the PG which has a great ecdysone precursor pool. The first in vivo ecdysone peak was seen on day 6 of 5th instar. EcR-B1 expression gradually decreased when compared with day 3 glands. This down-regulation may concern a too high concentration of 20E which PG exposed in vivo before dissection. The second large peak was seen on day 9 which induces larval-pupal ecydsis. EcR-B1 expression declined in a stepwise manner after day 9 and early pupal period. Intercellular spaces were clearly distinguishable after pupal ecdysis. PGs of insects degenerate during pupal period because the most adult insects do not molt (Sehnal et., al., 1996). Therefore our morphologic observations during prepupal and early pupal period indicated that programmed cell death process started in the early pupal stage. EcR-B1 expression was not significantly different between each experiment group, indicating that there is no more EcR-B1 needed for gland activity and other physiological process.

Juvenile hormone treatment groups

When day 0 glands were incubated with two different concentrations of juvenile hormone analogue fenoxycarb, similar immunolabelling was detected independent of treatment doses. In addition to this result, we did not find significant differences between control and fenoxycarb treatment groups. All of these results indicated that, fenoxycarb alone did not affect EcR-B1 expression status in PG. Persistence of immunoreactivity in fenoxycarb treated groups was determined on day 6, day 9 and day 10. When *M. sexta* epidermis was cultured with JH, it was found that JH maintained EcR-B1 protein presence (Hiruma et., al., 1999). Our results may support this report. EcR-B1 expression declined in a stepwise manner after day 10 like control but morphologic changes did not occur as clearly as in the control after pupation.

Ecdysone treatment groups

Ecdysone application to the culture medium reduced immunoreactivity of EcR-B1 when compared with control. Studies in the salivary glands of *D. melanogaster* (Huet et., al., 1995) and epidermis of *M. sexta* (Jindra et., al., 1996) showed that high concentrations of ecdysone caused down-regulation of EcR-B1 mRNA. Our results from ecdysone treatment groups may explain the negative feedback effect of ecdysone which may be involved in this inhibition. Unlike day 3 experiment, up-regulation of EcR-B1 expression was determined in day 6 treatment group but immunoreactivity was weaker than the control.

These results probably related to in vivo conditions before dissection, and additional ecdysone application to the culture medium caused a decline in EcR-B1 immunoreactivity when compared with the control. These results support the view that high ecdysone level downregulates EcR-B1 protein (Riddiford et al., 1999).

In addition to these results, immunoreactivity was dominantly determined in nucleus in day 3 treatment group. Nieva et al., (2007) demonstrated that the EcR is heterogeneously distributed between the cytoplasm and the nucleus in the absence of hormone. Lammerding-Köppel et al., (1998) reported that the presence of hormone increased nuclear localization of EcR in cell line from *Chironomus tentans*. According to these reports, 20E application caused nuclear transportation of EcR-B1 to the nucleus in this experiment group.

Fenoxycarb + ecdysone treatment groups

Two different doses of fenoxycarb (10ng/ml and 100ng/ml) were applied with one dose of 20E (0.5μg/ml) to the glands culture medium, and similar expression pattern was found in both of two groups. EcR-B1 expression was inhibited in day 0 treatments independent of fenoxycarb dose, but same inhibition were not observed if only JH was added on this day. Nagata et al. (1987) reported that low quantity of ecdysone is needed to response to another juvenile hormone analogue methoprene during larval period. This result suggests that inhibition of the EcR-B1 expression by fenoxycarb on day 0 occurred with the presence of ecdysone in the culture medium. The intensity of EcR-B1 signal increased in day 3 treatment groups when compared with day 0 glands. This result showed that fenoxycarb is not capable of inhibiting EcR-B1 expression on this day. Hiruma et al. (1999) reported that JH application to culture medium does not prevent the increase of EcR-B1 mRNA in response to ecdysone. In addition, this result also fazed us that juvenile hormone presence in the culture medium blocked the downregulation effect of ecdysone on the EcR-B1 expression.

Our study demonstrates that EcR-B1 is present in prothoracic glands of *B. mori*. PG secretes ecdysone in response to PTTH signal but especially in the last larval instar; there are some other factors such as its hormone, ecdysone, and JH that can also play important roles for its secretory activity status. For this reason, PGs also become a target of ecdysone. Gilbert et al. (1997) reported that an adequate amount of EcR complex is determined in the prothoracic glands of *M. sexta* only when the hemolymph ecdysteroid titer is high. In *B. mori*, hemolymph ecdysteroid concentration begins to rise on day 3 and two peaks are seen on day 6 and day 9 of fifth instar. EcR-B1 expression status in PGs is closely related to ecdysone and JH levels. High ecdysone concentrations reduced EcR-B1 protein expression. The differences of the means of immunoreactivity for the ecdysone application groups were statistically significant ($p \leq 0.05$) (Fig 7). It seems that fenoxycarb alone was not effective in the inhibition of EcR-B1 expression, but it may suggest that its presence stabilized EcR-B1 protein. Application of ecdysone and JH together, generally exerted the average of both effects. Gilbert et al (1997) suggested that ecdysone receptor complex of PG may regulate the secretion activity of glands by arranging the expression of proteins which were involved in ecdysteroid biosynthesis. From these results, negative feedback effect of ecdysone on secretory activity of the gland may be related to ecdysone receptor B1 and it may involve consistent and regular ecdysone secretion during fifth instar.

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