

EFFECTS OF A FAT BODY EXTRACT ON LARVAL MIDGUT CELLS AND GROWTH OF LEPIDOPTERA

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SUMMARY

Treatment with fat body extract (FBX) from pupae of the tobacco hornworm, *Manduca sexta*, caused mortality in larvae of two pest lepidopterans, the gypsy moth, *Lymantria dispar*, and the cotton leafworm, *Spodoptera littoralis*. In FBX-treated larvae, the feeding rate was depressed, causing reduced weight gain and then larval death. Their midgut showed formation of multicellular layers of midgut epidermis, indicating stem-cell hyperplasia. Hence, the integument of FBX-treated larvae had a double cuticle, indicating induction of premature molting. But radioimmunoassay measurements confirmed that the amount of ecdysteroids in FBX was too low to be responsible for the molt-inducing effects observed after treatment with FBX. With midgut stem cell cultures in vitro, addition of FBX to the culture medium stimulated cell proliferation and differentiation in a concentration-dependent manner. This effect was compared with those of insect molting hormones, ecdysone and 20-hydroxyecdysone; an ecdysteroid agonist, RH-2485; and a purified protein from FBX (multiplication factor). This article describes the mode of action of FBX and possible interplay between fat body factor(s) and insect hormones in the development and metamorphosis of the insect midgut.

Key words: midgut stem cells; 20-hydroxyecdysone; metamorphosis; hyperplasia; *Lymantria dispar*; *Spodoptera littoralis*.

In insects, growth and metamorphosis are governed largely by hormones and growth factors. When these bind to their specific receptors, changes such as cell division, differentiation, tissue repair, chemotaxis, cell death, and other processes are regulated (Hogan et al., 1994; Slack, 2000). Insect molting hormones, the ecdysteroids, induce metamorphosis in vivo and in vitro (Oberlander and Fulco, 1967; Akai, 1976; Riddiford, 1985; Smagghe et al., 1996), and insect growth factors also regulate insect development (reviewed by Loeb et al., 1999; Homma et al., 2001). The physiologically active molting hormone in most insects is 20-hydroxyecdysone (20E). However, products of fat body tissue also play a role in insect development. Oberlander and Tomblin (1972) and Benson et al. (1974) reported that a fat body component stimulated the development of lepidopteran imaginal disks cultured in medium containing 20E and fat body. Loeb and Hakim (1991) and Loeb (1994) reported that an aqueous extract of isolated fat body (FBX) from abdomens of newly ecdysed pupae of the tobacco hornworm, *Manduca sexta*, played a role in the initiation of meiosis in cultured sperm of the boll worm, *Heliothis virescens* (Giebultowicz et al., 1987), promoted growth and development of the male genital tract in *H. virescens* and the gypsy moth, *Lymantria dispar*, in vitro (Loeb and Hakim, 1991), and induced mitosis in cultured midgut cells of *M. sexta* (Sadrudin et al., 1994) and *H. virescens* (Loeb and Hakim, 1996). FBX also increased the growth-stimulatory effect of 20E

in vitro on imaginal wing disks of another lepidopteran, *Spodoptera littoralis* (Smagghe et al., 2001b). However, administration of as little as 1% FBX to intact last-instar larvae of *S. littoralis* inhibited larval growth and caused death before pupation (Smagghe et al., 2001b). The mechanism of action of FBX is not yet known.

In this study, we investigated the effect of FBX on growth and differentiation of midgut stem cells from two important lepidopteran pests, the gypsy moth, *L. dispar*, and the cotton leafworm, *S. littoralis*. In numerous countries, populations of both species have developed high degrees of pesticide resistance. *L. dispar* larvae were raised on artificial diet at 30° C in a 12:12 dark-light cycle; *S. littoralis* larvae were kept at 23° C and in a 16-h light cycle. Larvae were raised on artificial diet, and adults were provided with 15% honey water as described by Bell et al. (1981) and Smagghe et al. (2000). Fat bodies were excised from abdomens of newly molted *M. sexta* pupae and its extract was prepared as described by Loeb and Hakim (1996); aliquots of FBX were kept frozen at -20° C until use. Doses of FBX were administered to larvae of *L. dispar* and *S. littoralis* mixed in diets and by direct oral injection (in 5- μ l aliquots) using a Hamilton microsyringe (Bonaduz, Switzerland). Larval weights were recorded throughout development. In addition, midguts of FBX-treated larvae were dissected and fixed in Bouin's fluid, embedded in wax, sectioned, and stained using Carazzi's hematoxylin (Carazzi, 1911). The ecdysteroid content of FBX was compared with titers of ecdysteroids in the circulating hemolymph of control and FBX-treated larvae by standard radioimmunoassay (Gelman et al., 1997). We also evaluated the effects of the two

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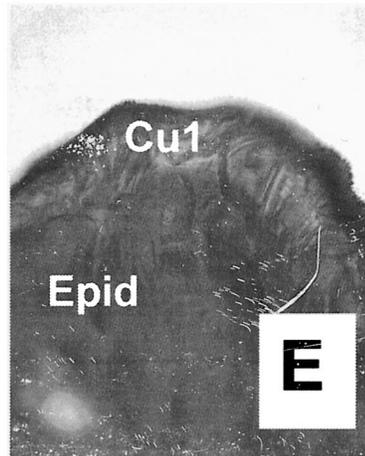
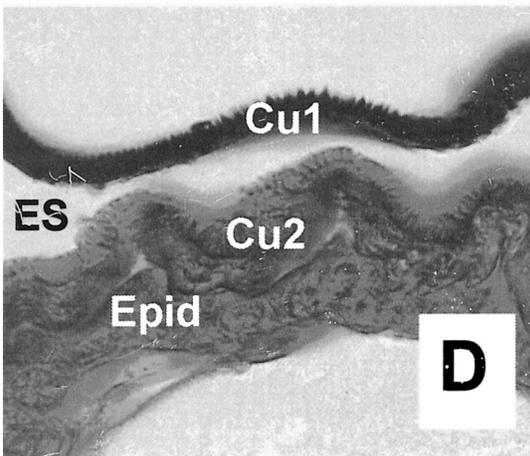
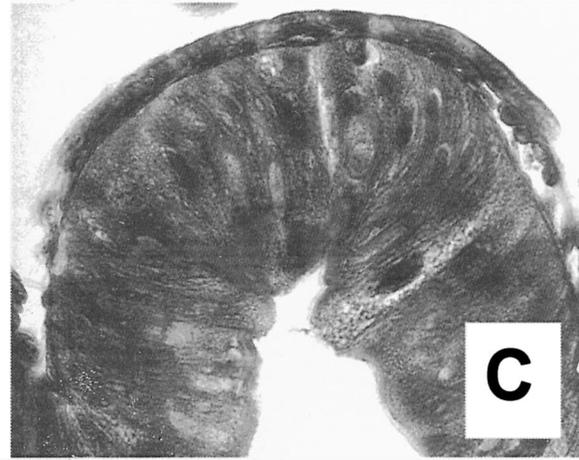
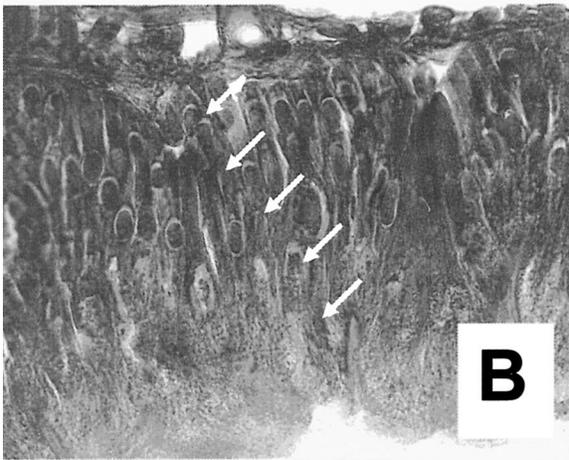
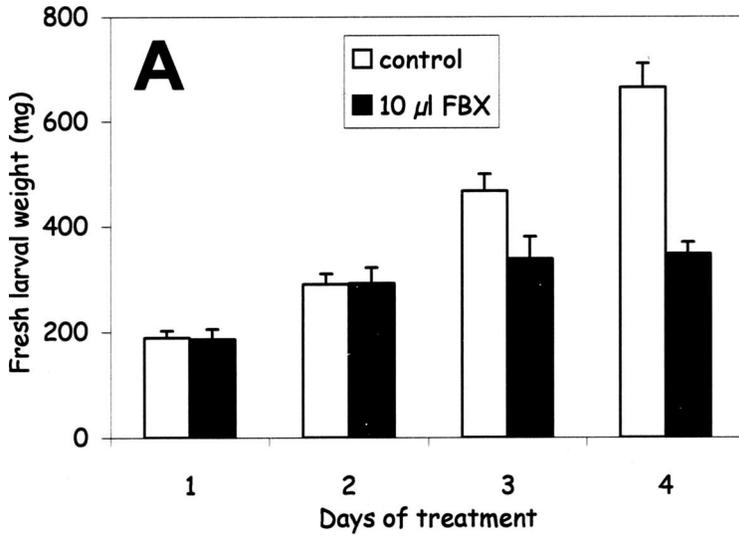


FIG. 1. (A) Fat body extract (FBX) inhibited larval growth and feeding in last-instar (sixth) larvae of *S. littoralis* at 10 µl when fed orally; n = 20; data are expressed as fresh weight means ± SD. In such FBX-treated larvae, transverse midgut sections demonstrated under the light microscope that treatment with 10 µl FBX stimulated cell proliferation in the midgut epithelium, leading to a multicellular layer and indicating stem cell hyperplasia (B) as compared with controls (C). Arrows show the formation of more than one layer of midgut epithelium, indicating stem cell hyperplasia after treatment with FBX. Transverse thoracic integument sections of FBX-treated larvae (D) demonstrated the presence of a double cuticle with the synthesis of a new cuticle (Cu2) by the epidermal cells (Epid) under the ecdysial space (ES) and the old cuticle (Cu1), suggesting the induction of molting/metamorphosis after treatment with FBX. Control larvae (E) were in intermolt phase with one cuticle (Cu1) present.

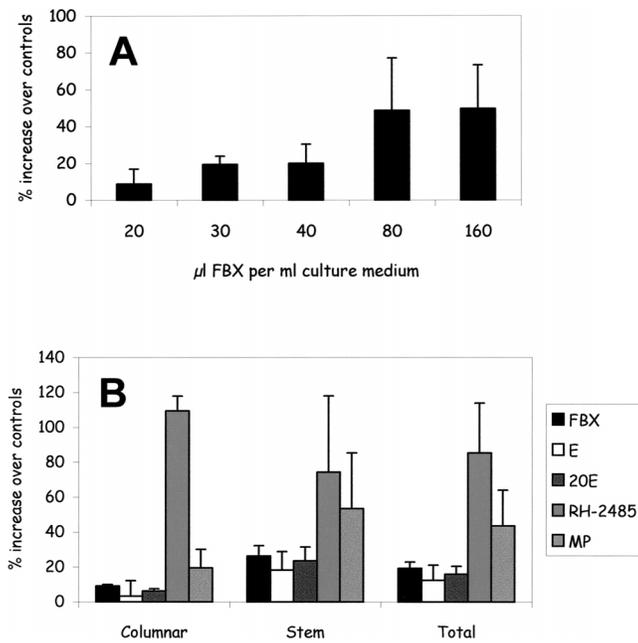


FIG. 2. (A) Increase in total midgut cell numbers over the control cultures after the addition of different concentrations of fat body extract (FBX) (20–160 $\mu\text{l/ml}$) to the culture medium. (B) Increase in columnar, stem, and total cell numbers over the control cultures after the addition of FBX (30 $\mu\text{g/ml}$), ecdysone (E, 2.2 pg/ml ; 1 μM), 20-hydroxyecdysone (20E, 2.1 pg/ml ; 1 μM), RH-2485 (2.7 pg/ml ; 1 μM), and multiplication factor (MP, 248 ng/ml) (248 ng/ml) to the culture medium. In both experiments, midgut stem cell cultures were prepared from fourth-instar larvae of *L. dispar* that exhibited head capsule slippage. Data were collected from four to eight replications and expressed as mean percentages \pm SEM of increase over the untreated control cultures.

principle ecdysteroids 20E and ecdysone (E) (both from Sigma Chemical Co., St. Louis, MO) and a metabolically stable ecdysteroid agonist RH-2485 (methoxyfenozide) (Rohm and Haas Co., Spring House, PA) on midgut stem cells in vitro. Previously, RH-2485 was found to have a high accumulation rate in target cells and high binding affinity to ecdysteroid receptors in Lepidoptera compared with ecdysteroids (Yund and Osterbur, 1985; Smagghe et al., 1999, 2001a).

FBX administered at 100 μl per larva (incorporated into 10 ml of diet) induced 50% mortality in third-instar larvae of *L. dispar*. Transverse sections of excised control midgut revealed the presence of the three major typical cell types: columnar cells, goblet cells, and small regenerative stem cells near the basal membrane of the monolayer of midgut epidermis, arranged in a typical longitudinal pattern (Billingsley and Lehane, 1996). Treatment with FBX provoked cell proliferation, resulting in a thick, randomly organized epithelial layer containing many columnar cells. Last-instar (sixth) larvae of *S. littoralis* were more susceptible to FBX: only 10 μl of FBX incorporated into the diet or injected orally was sufficient to strongly depress the feeding rate, causing less weight gain than observed for controls (Fig. 1A) and resulting in the death of all the treated *S. littoralis* larvae after 4–5 d. Inspection by dissection indicated disruption of the midguts in these dying caterpillars. Stained sections of the midguts of FBX-treated *S. littoralis* larvae showed formation of multicellular layers of midgut epidermis, indicating stem cell hyperplasia, loss of typical organization, and simultaneous production of more than one midgut

epithelium (Fig. 1B). In contrast, hyperplasia was never observed in the sections of intermolt larval midgut in the control group (Fig. 1C). The observations of these pathologies are strikingly similar to the descriptions of midguts of larvae of *Hyalophora cecropia* undergoing metamorphosis to pupae (Judy and Gilbert, 1969). Tanaka and Yukuhiro (1999) reported complete replacement of the midgut in *Bombyx mori* when larvae entered apolysis. Thus, it appeared that the midguts of FBX-treated caterpillars of *L. dispar* and *S. littoralis* underwent a process similar to the one that occurs during metamorphosis. We were also surprised to observe that the integument of FBX-treated larvae of *S. littoralis* was composed of a double cuticle (Fig. 1D), which may confirm the induction of molting/metamorphosis by FBX treatment.

Radioimmunoassay of FBX (Gelman et al., 1997) demonstrated that 10 μl of FBX contained about 300 ± 11 pg of 20E equivalents. With different doses of FBX, 5–100 μl , we estimated a linear positive curve, $Y = 17.86X + 191.00$, with $R^2 = 0.91$, where Y = ecdysteroids mass (picograms), and X = FBX volume (microliters). The ecdysteroid titer of the circulating hemolymph of last-instar larvae of *S. littoralis* was 1.2 ± 0.2 $\text{pg}/\mu\text{l}$ (2.5 ± 0.5 nM) and 8.2 ± 3.0 $\text{pg}/\mu\text{l}$ (17.0 ± 8.3 nM) for control larvae and for larvae treated with 10 μl of FBX, respectively. The normal peak level of ecdysteroids at apolysis in vivo was previously measured at >100 $\text{pg}/\mu\text{l}$ (200 nM) (Smagghe et al., 2000). Although FBX provoked a small increase in the endogenous ecdysteroid titer in susceptible larvae, it was not enough to cause ecdysteroid-induced metamorphosis. Injection of 3 ng of 20E through the cuticle into the circulating hemolymph of last-instar larvae of *S. littoralis* had no molt-inducing activity, although 10 ng induced ecdysone effects in 40% of the larvae. On injecting a dose of 100 ng 20E, all larvae underwent premature molting. Therefore, it was clear that the amount of ecdysteroid in FBX was too low to be responsible for the molting/metamorphosis effects observed in the midgut after treatment with FBX. Smagghe et al. (2001b) demonstrated that FBX potentiated the action of 20E to induce imaginal disk development in vitro. Because 20E alone at high concentration produced effects similar to those of FBX in vivo, it is possible that 20E may have induced the production of the active factor of FBX by the fat body; this factor may be taken up by the midgut to induce stem cell multiplication and, in cases of oversupply of FBX, hyperplasia. We, therefore, speculate that the action of FBX on the midgut may be related and perhaps synergistic to the action of ecdysteroids in inducing molting/metamorphosis in larvae of *L. dispar* and *S. littoralis* and that ecdysteroids may also play a role in stimulating the production of the active factor of FBX.

Cultures of the midguts of late fourth-instar *L. dispar* larvae were prepared to study the phenomenon of FBX's action more closely. Larvae were surface sterilized, and midguts were dissected into culture medium consisting of modified Grace's medium (Life Sciences, Grand Island, NY) with 7% fetal calf serum (Life Sciences) and 30 fg/ml 20E (Sigma) (Loeb and Hakim, 1996; Loeb et al., 1999). The midguts were incubated in the medium for 1 h before they were removed; the stem cells that had migrated from the midgut tissue remained in the medium, which consisted of 50–75% stem cells (Loeb et al., 1999). Five hundred midgut stem cells were cultured in 400 μl of culture medium per well in a sterile 24-well culture plate (Becton Dickinson, Franklin Lakes, NJ) (Loeb et al., 2003). The total number of columnar, goblet, and stem cells per well were counted after 72 h of culture at 26° C. FBX at 20–160 $\mu\text{l/ml}$ increased cell numbers in a dose-dependent manner (Fig. 2A). Figure 2B shows that 30 μl of FBX per milliliter of the medium caused an increase of $19.3 \pm 4.6\%$ of the total number

of cells over the controls, including a $26.3 \pm 8.1\%$ increase in stem cells and $9.2 \pm 1.2\%$ increase in columnar cells. The effect was similar to that produced by addition of 20E at 2.1 pg/ml (1 μ M). Total cell numbers showed an increase of $15.9 \pm 8.7\%$, stem cells $23.5 \pm 10.6\%$, and columnar cells $6.3 \pm 8.9\%$ over the control cultures. The increase in the presence of E at 2.2 pg/ml (1 μ M) tended to be lower, but not significantly lower, than the increase observed when 20E was added to the stem cell cultures (Fig. 2B). Although E is considered a prohormone that is secreted by the prothoracic glands in insects and converted to the actual molting/metamorphosis hormone 20E, in insect tissues (Rees, 1985; Smith, 1985), the effect of E on midgut cell proliferation in our experiments was of the same order of magnitude as that of 20E at the doses used. In addition, it was striking that the metabolically stable and rapidly accumulated ecdysteroid agonist RH-2485 at 2.7 pg/ml (1 μ M) induced an even greater increase in total cell number, $85.3 \pm 28.5\%$. This dramatic increase was seen in columnar and stem cells, which increased $109.3 \pm 8.5\%$ and $74.3 \pm 43.6\%$ over the control cultures, respectively (Fig. 2B). In vivo, ecdysteroids, but not RH-2485, are subjected to rapid metabolic degradation, which may account for the powerful effect of RH-2485 on stem cell differentiation and multiplication (Rees, 1985; Smaghe et al., 1999, 2001a). FBX had approximately the same effect on cell numbers as 20E at the doses applied. Loeb and Hakim (1991) and Sadrud-din et al. (1994) had shown that both FBX and 20E were essential for mitosis of *H. virescens* and *M. sexta* midgut stem cells in vitro.

In continuation of our study, we tested a purified protein isolated from FBX (multiplication factor [MP]) that appears to be responsible for the multiplication of midgut cells in vitro (M. Blackburn, pers. comm.). In midgut stem cell cultures from *L. dispar*, MP at 248 ng/ml of medium caused an increase of $43.5 \pm 20.5\%$ in total cells over the untreated controls (Fig. 2B). This response is similar to the one induced by 40–80 μ l of FBX per milliliter of the medium (Fig. 2A). Recombined fractions of FBX in which MP had been removed did not have any effect on cell multiplication and differentiation in midgut stem cell cultures; treatment with up to 40 μ l/ml of culture medium resulted in the same numbers of columnar, stem, and total cells as in the control cultures. The characterization of MP is in progress.

During molting and metamorphosis, development of the midgut must be tightly regulated. There is surely a delicate and complex interplay of multiple factors including those that originate from different tissues, such as the fat body and multi-action hormones such as ecdysteroids. Because 20E alone produced effects similar to those of FBX in vitro and in vivo, albeit at higher concentrations, it is likely that these molecules act together in an intact animal. In addition, it is possible that 20E induces the production of MP by the fat body; MP may be taken up by the midgut to induce stem cell multiplication in vivo. The death of the FBX-treated larvae of *L. dispar* and *S. littoralis* during development was probably provoked by a disruption of endocrine function, inhibition of feeding due to a disrupted midgut structure, or both. Additional in vivo and in vitro experiments are in progress in our laboratory to sort out the interrelationship of factors that induce midgut cell multiplication.

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