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REGULATION OF CYTOCHROME P-450 DEPENDENT STEROID HYDROXYLASE ACTIVITY IN *MANDUCA SEXTA*: EVIDENCE FOR THE INVOLVEMENT OF A NEUROENDOCRINE-ENDOCRINE AXIS DURING LARVAL-PUPAL DEVELOPMENT

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SUMMARY. Ecdysone 20-monooxygenase activity and the factors which may regulate this steroid hydroxylase were examined in the midgut of the tobacco hornworm, *Manduca sexta*, during the last larval stadium. Radioassay experiments revealed that midgut ecdysone 20-monooxygenase undergoes a single 50-fold increase in activity temporally coincident with the onset of the wandering stage. The increase in midgut monooxygenase activity was prevented by actinomycin D and cycloheximide, and could be elicited in head (but not thoracic) ligated animals by a brain-retrocerebral complex factor(s) released at the same time as prothoracicotropic hormone. In contrast, ecdysone or 20-hydroxyecdysone could elicit the increase in enzyme activity in both head and thoracic ligated animals. These data suggest the operation of a neuroendocrine-endocrine axis in the regulation of midgut ecdysone 20-monooxygenase activity.

INTRODUCTION. *In vivo* studies have suggested that the conversion of the insect molting hormone ecdysone (2ß,3ß,14α,22R,25-pentahydroxy-5ß-cholest-7-en-6-one) to its more active metabolite 20-hydroxyecdysone varies considerably during the life cycle of insects (1). Given the importance of this reaction during insect postembryonic development, several investigations have focused on the characterization of the enzyme system, ecdysone 20-monooxygenase (EC 1.14.99.22), responsible for this hydroxylation. These studies have revealed that ecdysone 20-monooxygenase is essentially an NADPH requiring cytochrome P-450 dependent steroid hydroxylase similar to those present in vertebrate steroidogenic tissues (1-5). In accord with the earlier *in vivo* studies, more recent *in vitro* measurements of ecdysone 20-monooxygenase activity have confirmed that this steroid hydroxylase activity fluctuates dramatically, and in some cases in a tissue specific fashion, during insect postembryonic development and adult life (1, 5-8).

The dramatic, temporally precise and tissue specific fluctuations in ecdysone 20-monooxygenase activity suggest that this cytochrome P-450 dependent steroid hydroxylase may itself be under some form of regulation. Several studies have implicated ecdysone and 20-hydroxyecdysone as possible regulators of ecdysone 20-monooxygenase activity but it remains to be established whether or not such regulation is physiological in nature (1, 9, 10). Accordingly, in the present study we have examined ecdysone 20-monooxygenase activity and the factors which

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may regulate this enzyme activity in the midgut of the tobacco hornworm, *Manduca sexta*, during larval-pupal development.

MATERIALS AND METHODS. Animals. The animals used in this investigation were last instar larvae of the tobacco hornworm, *Manduca sexta*. Animals were reared on an artificial diet under a non-diapausing photoperiod (L:D, 16:8) at 25°C and 60% relative humidity (11). Animals were staged as previously described (12) and only gate II fifth instar larvae or day 1 pupae were used in these studies.

Ecdysteroids and Chemicals. The radiolabelled ecdysteroid substrate for the monooxygenase assay was [23,24-^{3}H]-ecdysone (stocks of 45 and 70 Ci/mmol) purchased from New England Nuclear, Boston, MA. Ecdysteroid standards, cycloheximide, NADPH, phenylmethylsulfonyl fluoride, phenobarbital, actinomycin D and bovine serum albumin (BSA) were purchased from Fluka Chemical Corp., Ronkonkoma, NY, and Sigma Chemical Co., St. Louis, MO; salts, organic solvents, and scintillation fluid (ScintiVerse E) were purchased from Fisher Scientific Co., Cleveland, OH; cholesterol was purchased from United States Biochemical Corp., Cleveland, OH.

Tissue Dissection and Homogenization. Midgut from gate II larvae and brains from day 1 pupae were dissected in a lepidopteran Ringer's at 4°C (13). Homogenates of midgut were made at 10 to 100 mg/ml in sodium phosphate buffer (50 mM, pH 7.5, containing 250 mM sucrose) using a Potter-Elvehjem tissue grinder with a motor driven Teflon pestle (275 rpm, 20 strokes, 0-4°C). Day 1 pupal brains (including the retrocerebral complex) were rinsed in the above Ringer's, homogenized at 200 brain equivalents/ml, and sonically disrupted (3 x 30 sec bursts, 20W) using a Branson Model S110 Sonifier equipped with a microtip.

Animal Ligations and Injections. Animals to be ligated or injected were chilled on ice. Head (between head and prothoracic segment) and thoracic (between metathorax and first abdominal segment) ligations were carried out using waxed dental floss. Head ligated animals had the anterior portion of the head capsule and brain-retrocerebral complex removed; thoracic ligated animals had the head and thoracic regions removed (i.e., these animals were essentially isolated abdomens). All injections into whole, head ligated, or thoracic ligated animals were given via the abdominal prolegs using 10 or 50 ul Hamilton syringes and the injection sites ligated with waxed dental floss; untreated and sham injected animals served as controls. Brain-retrocerebral complex homogenates were injected into animals on day 3 (plus 0 hr) of the stadium, total injection volume was 25 ul (5 brain equivalents). Cycloheximide (0 to 50 ug) and actinomycin D (12.5 or 25 ug) were injected into day 3 (plus 0 hr) or day 4 (plus 4 hr) animals, total injection volume was 10 ul. Cholesterol and phenobarbital at stock concentrations of 3.5 x 10^{-2} M were injected into animals on day 3 (plus 0 hr) of the stadium; total injection volume was 1 ul per gm weight of animal. Ecdysone and 20-hydroxyecdysone were administered into the animals as single injections (0.07 to 3.0 ug total per gm wt) on day 3 (plus 0 hr), double injections (2.8 to 6 ug total per gm wt) on day 3 (plus 0 hr and plus 8 hr), or triple injections (8.4 to 16.8 ug total per gm wt) on day 3 (plus 0 hr, plus 8 hr and plus 20 hr), injection volumes were all less than 10 ul per animal.

Ecdysone 20-Monooxygenase Assay. Ecdysone 20-monooxygenase activity was detected and quantified in the midgut using a radioassay (4). For the assay, 0.05 ml aliquots of midgut homogenate (containing from 0.5 to 5 mg tissue equivalent)were added to 0.05 ml aliquots of 0.05 M sodium phosphate buffer, pH 7.5, containing [23,24-^{3}H] ecdysone (0.5 to 15.2 ng; 1.2 to 70 Ci/mmol; 0.11 to 3.3 x10^{-7}M assay concentration) and NADPH (1.6 x10^{-3}M assay concentration). Some assays also contained phenylmethylsulfonyl fluoride (1.0 x10^{-3}M assay concentration), BSA (0.5 mg/assay) or both. Assay incubations were for 30 min at 30°C with constant agitation. All assays were run in duplicate with zero time controls and were terminated by the addition of 1.5 ml ethanol. Following termination, assay mixtures were centrifuged at 10,000g for 10 min and 0.15 ml aliquots of the assay supernatant plus 2 ug each of cold carrier ecdysone and 20-hydroxyecdysone were evaporated to dryness. The residues were redissolved in methanol and streaked on analytical thin layer chromatography (TLC) plates (0.25 mm silica gel 60, F-254; E. Merck, Darmstadt, Germany). The plates were developed in a solvent system of chloroform: 95% ethanol (1:1, v/v) and the ecdysone and 20-hydroxyecdysone bands visualized under short wavelength UV light. The ecdysteroid bands were scraped into scintillation vials, resuspended in scintillation fluid and counted using a Beckman Model 3801 scintillation counter (^{3}H counting efficiency, 65%). Control ecdysone 20-monooxygenase activity was expressed as pg 20-hydroxyecdysone (20-HE) formed/min/mg tissue (±SEM).

RESULTS AND DISCUSSION. Radioassay quantification of midgut ecdysone 20-monooxygenase revealed that this steroid hydroxylase activity fluctuates dramatically during larval-pupal development of *Manduca sexta* (Fig. 1). The midgut enzyme activity was basal (3 to 12 pg 20-HE/min/mg tissue) during the feeding and pre-wandering stage (days 0-4) of the fifth larval stadium. Concomitant within the onset of the wandering stage on day 5 the midgut ecdysone 20-monooxygenase activity increased approximately 50 fold to 393 (±18) pg 20-HE/min/mg tissue and remained elevated for the duration of the wandering stage. With the onset of pharate pupal development (day 7), midgut ecdysone 20-monooxygenase dropped sharply to 70 (±4) pg 20-HE/min/mg tissue before declining to basal levels for the remainder (days 8 to 10) of the last larval stadium (Fig. 1). This profile of midgut ecdysone 20-monooxygenase activity is in essential accord with previous reports (1,6) and confirms that this midgut enzyme is a potentially excellent model for investigating the regulation of insect cytochrome P-450 dependent steroid hydroxylase systems.

Given the physiologically significant 50-fold increase in midgut ecdysone 20-monooxygenase activity on day 5 of the stadium, several parameters which might affect this steroid hydroxylase were examined. Inclusion of BSA (5 mg/assay) or phenylmethylsulfonyl fluoride

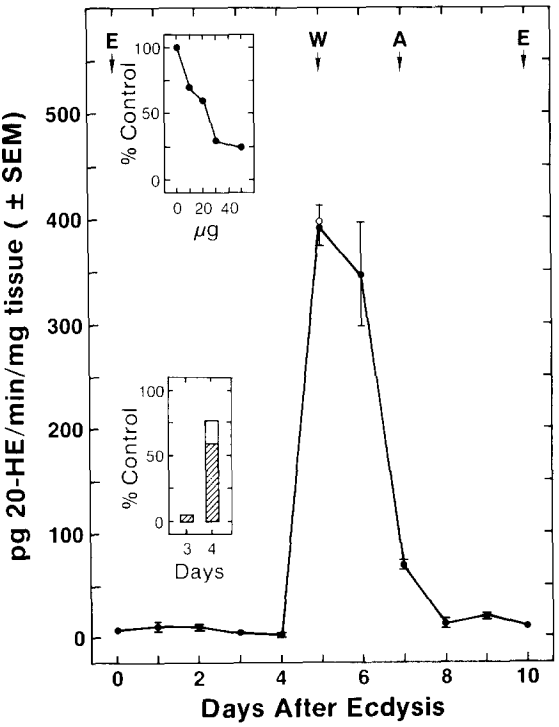

Fig. 1. Profile of midgut ecdysone 20-monooxygenase activity during the 10 day stadium of the last larval instar of *Manduca sexta* (closed circles, n = 4 to 84 assays for all data points; open circle depicts activity in day 3 plus day 5 tissues mixed, n = 6 assays). E, ecdysis; W, onset of wandering stage; and A, apolysis and onset of pharate pupal development. Top inset depicts the effects of cycloheximide (injected on day 3) on day 5 midgut ecdysone 20-monooxygenase activity (n = 4 assays for all data points; control enzyme activity, 387 pg 20-HE/min/mg tissue). Bottom inset depicts the effects of actinomycin D (injected on day 3 or day 4 plus 4 hr) on day 5 midgut ecdysone 20-monooxygenase activity (n = 4 assays for both bars; open bar equals 12.5 ug dose, striped bar equals 25 ug dose; control activity, 416 pg 20-HE/min/mg tissue).

($1x10^{-3}$ assay concentration) or both in the assay did not elicit any increase in the enzyme activity associated with day 3 or day 5 midgut tissues. Furthermore, incubation of day 3 with day 5 midgut tissues revealed that the total assay monooxygenase activity was additive (Fig. 1). These data suggested, therefore, that the dramatic increase in midgut steroid hydroxylase activity is neither a reflection of the loss of any endogenous protease activity on day 5 of the stadium nor the loss of any endogenous inhibitors of cytochrome P-450 dependent enzyme activity (14).

In an attempt to determine whether the increase in midgut ecdysone 20-monooxygenase activity is dependent on protein synthesis or gene transcription, the effects of cycloheximide and actinomycin D were examined. Injections of cycloheximide into day 3 (plus 0 hr) larvae prevented in a dose-response fashion the subsequent increase in midgut monooxygenase activity (Fig. 1, top inset). Animals receiving 50 ug of cycloheximide exhibited a mean level of midgut ecdysone 20-monooxygenase activity on day 5 that was only 25% that of sham injected controls. Actinomycin D injections also prevented the 50-fold increase is midgut ecdysone 20-monooxygenase activity (Fig. 1, bottom inset). Injections of 12.5 or 25 ug of actinomycin D into day 3 (plus 0 hr) larvae completely prevented any subsequent increase in midgut monooxygenase activity on day 5, while animals receiving such injections on day 4 (plus 4 hr) exhibited sustantially lower midgut ecdysone 20-monooxygenase activities on day 5 compared to sham injected controls. These data provided evidence that both protein synthesis and gene transcription are required, either directly or indirectly, for the 50-fold increase in midgut steroid hydroxylase activity.

Further studies involving head ligation experiments demonstrated that a head factor(s), released between day 3 (plus 12 hr) and day 4 (plus 4 hr) of the stadium, is necessary for the subsequent increase in midgut steroid hydroxylase activity on day 5 of the stadium (Fig. 2). Interestingly, the head critical period (HCP) for this hydroxylase activating head factor(s) was temporally coincident with that for the major neurohormone which regulates insect postembryonic development, viz., prothoracicotropic hormone or PTTH (1). To test in part whether this hydroxylase activating head factor might be PTTH or at least brain derived, day 1 pupal brain-retrocerebral complexes, a rich source of PTTH (15), were sonicated and injected into day 3 (plus 0 hr) head or thoracic ligated animals (Fig. 2, inset). Data from these experiments revealed that injections of these sonicates into day 3 (plus 0 hr) animals elicited the 50-fold increase in midgut steroid hydroxylase activity in about 75% of the head ligated animals, but were totally ineffective when injected into isolated abdomens. This latter failure in isolated abdomens demonstrated that the brain factor is not directly responsible for the increase in midgut ecdysone 20-monooxygenase activity and suggested that additional factors, most probably from the thoracic region, are also required for midgut enzyme activation.

Working under the assumption that PTTH is the brain hydroxylase activating factor, the possibility was tested that ecdysone or its metabolite 20-hydroxyecdysone might be the thoracic factor directly responsible for eliciting the 50-fold activation of midgut ecdysone 20-monooxygenase activity. Previous studies with last instar larvae of *Manduca sexta* have demonstrated not only that PTTH is released during the time of our HCP for the increase in midgut monooxygenase activity, but that in response to PTTH the prothoracic glands synthesize and release ecdysone (some of which is converted by the fat body to 20-hydroxyecdysone) in a pulsatile manner on late day 3-early day 4 of the stadium (1). Accordingly, increasing amounts of

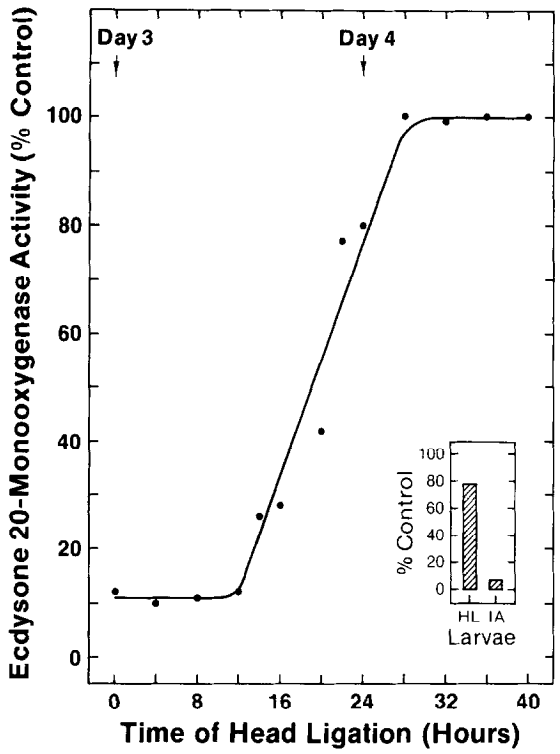

Fig. 2. Effects of prior head ligation on day 5 midgut ecdysone 20-monooxygenase activity. Animals were head ligated at the times indicated, decapitated and the midgut enzyme activity subsequently quantified on day 5 (n = 4 to 18 assays for all data points; untreated day 5 control monooxygenase activity, 373 pg 20-HE/min/mg tissue). Inset depicts day 5 midgut ecdysone 20-monooxygenase activity (n = 4 assays for both bars; control, 341 pg 20-HE/min/mg tissue) in animals head ligated (HL) or thoracic ligated (IA, isolated abdomen) and injected with five day 1 pupal brains on day 3 of the stadium.

ecdysone or 20-hydroxyecdysone were injected into head or thoracic ligated animals on day 3 (plus 0 hr, plus 8 hr, plus 20 hr) and the midgut monooxygenase activity quantified on day 5. In contrast to brain sonicates, increasing amounts of ecdysone (Fig. 3) or 20-hydroxyecdysone (Fig. 3, inset) where found to elicit dose response increases in midgut ecdysone 20-monooxygenase activity in both head and thoracic ligated animals; on a per molar basis, ecdysone was about twice as effective as 20-hydroxyecdysone. The activating effects of ecdysone and 20-hydroxyecdysone appeared to be specific since phenobarbital, an inducer of b and e forms of cytochrome P-450 monooxygenases (16), and cholesterol were totally ineffective in eliciting any increase in midgut ecdysone 20-monooxygenase activity even when injected into head or thoracic ligated day 3 animals at higher concentrations than the ecdysteroids.

Although pharmacological amounts of ecdysone or 20-hydroxyecdysone were required to elicit the 50-fold increase in midgut ecdysone 20-monooxygenase activity, there are reasons for suspecting that this may be a reflection of either the delivery mode or the rapid catabolism of exogenously administered ecdysteroids (1). Infusion of ecdysteroids into *Manduca sexta* larvae has been shown previously (17) to elicit wandering stage behavior at much lower concentrations than those utilized in this study and, to date, all animals in our laboratory exhibiting wandering stage behavior have also exhibited increased midgut steroid hydroxylase activity. In addition,

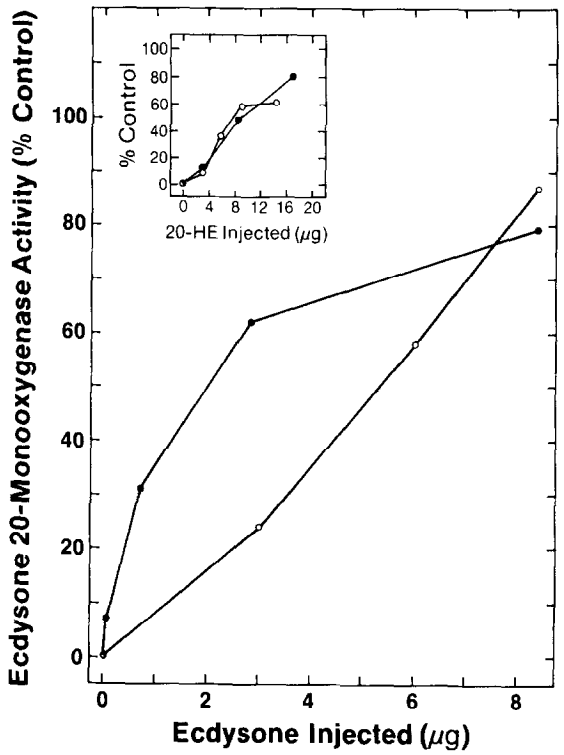

Fig. 3. Day 5 midgut ecdysone 20-monooxygenase activity in animals head ligated (closed circles) or thoracic ligated (open circles) on day 3 of the stadium and injected with increasing amounts of ecdysone or 20-hydroxyecdysone (20-HE; inset). Untreated day 5 control monooxygenase activity for the ecdysone experiments was 444 pg 20-HE/min/mg tissue (n = 2 to 15 assays for all data points), and for the 20-hydroxyecdysone experiments was 401 pg 20-HE/min/mg tissue (n = 2 to 5 assays for all data points).

more recent experiments in our laboratory with the ecdysone agonist RH 5849 (18) have revealed that this hydrazine (probably as a function of its lower rate of catabolism or excretion than ecdysteroids) can elicit the 50-fold increase in midgut enzyme activity at concentrations much lower than ecdysone and 20-hydroxyecdysone (19).

In conclusion, our data collectively support the model that the increase in midgut ecdysone 20-monooxygenase activity is physiologically regulated during larval-pupal development of *Manduca sexta*; this regulation probably involves the components of a neuroendocrine-endocrine axis (PTTH→ prothoracic glands→ ecdysone→ midgut). Our data, therefore, confirm and establish the physiological basis for earlier studies demonstrating an "induction" of ecdysone 20-monooxygenase activity in *Locusta migratoria* and *Musca domestica* by ecdysteroids (1,9,10). It is also clear from our study that the increase in midgut steroid hydroxylase activity is predicated on gene transcription and protein synthesis. Whether these transcriptional and translational events are involved directly with components of the midgut steroid hydroxylase system or indirectly, involving perhaps regulatory steroid hydroxylase inducing proteins (20) or post-translational modulatory proteins (21), remains to be determined.

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