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JUVENILE HORMONE-STIMULATED PROLIFERATION OF ENDOPLASMIC RETICULUM IN FAT BODY CELLS OF A VITELLOGENIC INSECT, Leucophaea maderae (Blattaria)

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SUMMARY. The rate of incorporation of [^{14}C]choline or [^{32}P]orthophosphate into phospholipids of rough and smooth-surfaced microsomal membranes from insect fat body was stimulated by juvenile hormone. The maximum incorporation rate *in vivo* occurred three days following hormone application. During *in vitro* incubation of the fat bodies from hormone-treated females [^{32}P] was taken up by the cells at about twice the rate of the controls, but its incorporation into microsomes was elevated 3.5 to five-fold. No difference in [^{32}P] precursor pools was observed *in vivo*. The enhanced proliferation of endoplasmic reticulum is temporally coupled to the hormone-induced *de novo* biosynthesis of an exportable protein, vitellogenin.

A well-developed endoplasmic reticulum (ER) is characteristic of cells that are actively synthesizing exportable proteins (1). The ER provides attachment sites for polyribosomes (2,3), and functions in post-translational modification of secretory products sequestered within the cisternae (4,5). Insect vitellogenins are juvenile hormone (JH) induced exportable proteins that are synthesized on membrane-bound polysomes in the fat body cells (6-9). Electron micrographs of fat body tissues from several species have revealed that well-developed arrays of stacked rough ER occur only in vitellogenic animals (10-13). Furthermore, microsomes harvested from the fat bodies of vitellogenic females are primarily ribosome-studded, whereas mostly smooth-surfaced vesicles are found in non-vitellogenic animals (7). From these observations we speculated that JH might be involved in the synthesis and assembly of ER membranes, and thus influence the rate of vitellogenin production by controlling the availability of new binding sites for vitellogenin polysomes.

We now report that JH stimulates the rate of incorporation of [^{32}P]orthophosphate and [^{14}C]choline into phospholipids of rough and

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smooth-surfaced microsomal membranes from fat body cells of the insect *Leucophaea maderae*.

MATERIALS AND METHODS

Surgical removal of the corpora allata, and the procedure for harvesting total fat body microsomes, has been previously described for *Leucophaea* (7). For sub-fractionation of microsomes into rough and smooth-surfaced populations, the 11,000 g post-mitochondrial supernatants of fat body homogenates were layered over a 1.08 M sucrose-TKM solution (50 mM Tris-HCl (pH 7.6), 25 mM KCl, 35 mM $KHCO_3$, and 10 mM MgAc) that was underlaid with a 2.0 M sucrose cushion. Following centrifugation at 149,000 g for 20 hrs (Beckman L2-65B, SW 50.1 rotor) the smooth (floating on the 1.08 M sucrose-TKM layer) and rough membranes (at the 1.08 M/2.0 M sucrose-TKM interface) were aspirated, suspended in TKM buffer, and pelleted at 149,000 g for 90 minutes. These membrane fractions had RNA/phospholipid ratios (µg/µg) of 0.42 and 0.03 for rough and smooth-surfaced microsomes respectively. A ten-fold difference in this ratio is thought to represent adequate separation of rough and smooth microsomes (14). Addition of cycloheximide (200 µg/ml) or emetine (25 µg/ml) to the homogenization and gradient media did not increase the yield of rough membranes, indicating that the generation of smooth microsomes via "run-off" is minimal. We are aware, however, that smooth microsomal fractions may be augmented by Golgi and plasma membrane-derived elements. Tissues radiolabeled *in vivo* or *in vitro* were washed in at least five changes of ice-cold insect saline, prior to homogenization, for removal of exogenous label.

Rates of ER synthesis were determined by measuring the incorporation of [methyl-^{14}C]choline (53.0 mCi/mmol, New England Nuclear) (15) or [^{32}P]orthophosphate (carrier-free, ICN) into microsomes. Total microsomal lipids were extracted in chloroform-methanol (2:1) according to Folch *et al.* (16) and washed in 0.73% NaCl or 0.04% $CaCl_2$. Total lipid phosphorus was determined according to Chen *et al.* (17). Membrane lipids were chromatographed on silica gel-H TLC plates with $CHCl_3$-MeOH-H_2O (70:30:4, v/v/v) as the developing solvent. Phospholipids were visualized with iodine vapor and identified by co-chromatography with phosphatidylcholine (PTC) and phosphatidylethanolamine (PTE).

Intracellular precursor pools of [^{32}P]orthophosphate were determined by radioassaying the phosphomolybdate-extractable material from the TCA-soluble post-microsomal supernatants of fat body homogenates (18). Total intracellular inorganic phosphate (Pi) was determined directly from an aliquot of the TCA-soluble material (17). Immunological procedures for determining rates of vitellogenin synthesis have been described elsewhere (19).

Juvenile hormone III (10-epoxy-3,7,11-trimethyl-2,6-trans, trans-dodecadienoic acid, methyl ester) was dissolved in either acetone or mineral oil and topically applied to the dorsal abdominal cuticle of the animals.

RESULTS

Rates of incorporation of [^{14}C]choline into fat body microsomal phospholipids (PL) were measured in allatectomized (CA^-) adult females that had received a single dose of JH III. After a one-day lag period, the rate of

choline incorporation had increased four- to five-fold by the third day after hormone administration (Fig. I). Both rough and smooth-membrane fractions were maximally labeled on day three, and thereafter declined to control values by day five. More than 90% of the lipid-extractable radioactivity was identified as PTC on silica gel TLC plates. We found that PTC was the most rapidly synthesized and quantitatively the major PL species in Leucophaea fat body microsomes (unpublished). The induction of vitellogenin synthesis following JH treatment was similarly associated with a one-day lag period. The maximum rate of synthesis occurred on day three and then gradually declined (Fig. I).

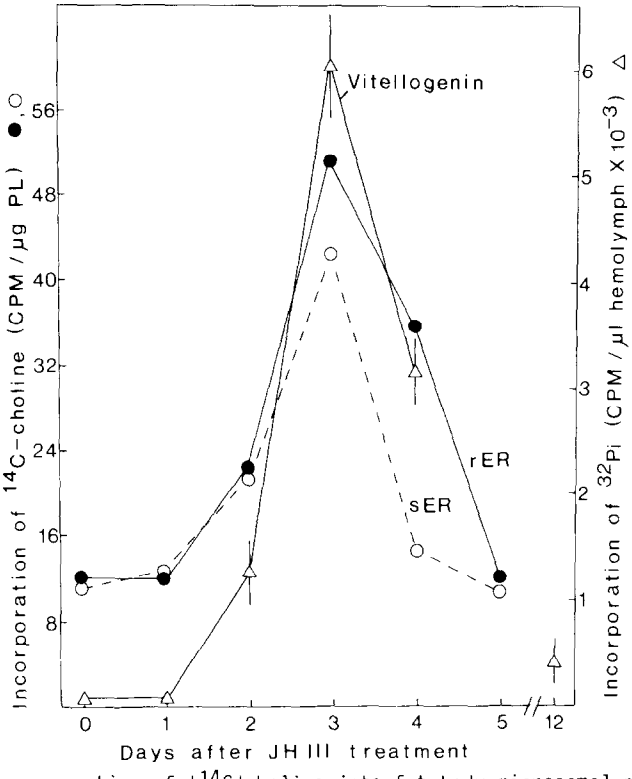

Figure I. Incorporation of [^{14}C]choline into fat body microsomal phospholipids and [^{32}P]orthophosphate into hemolymph vitellogenin. MICROSOMES: Four-week old CA$^-$ adult females received 40µg of JH III (Hoffmann-LaRoche) by topical application (dissolved in 2µl of mineral oil). Animals were injected with 2.0µCi of [^{14}C]choline for four hours and rough and smooth-surfaced fat body microsomes (rER and sER respectively) were isolated. Each point represents the microsomal phospholipid (PL) obtained from the pooled fat bodies of 3-5 animals. VITELLOGENIN: CA$^-$ females were treated with 100µg of JH III (Calbiochem) in 2µl acetone and labeled in vivo with 150µCi of [^{32}P] for three hours. Hemolymph was collected and immunoprecipitable vitellogenin was dissolved in 1.0N NaOH for radioassay (19). The bars represent the mean ± standard error based on three determinations.

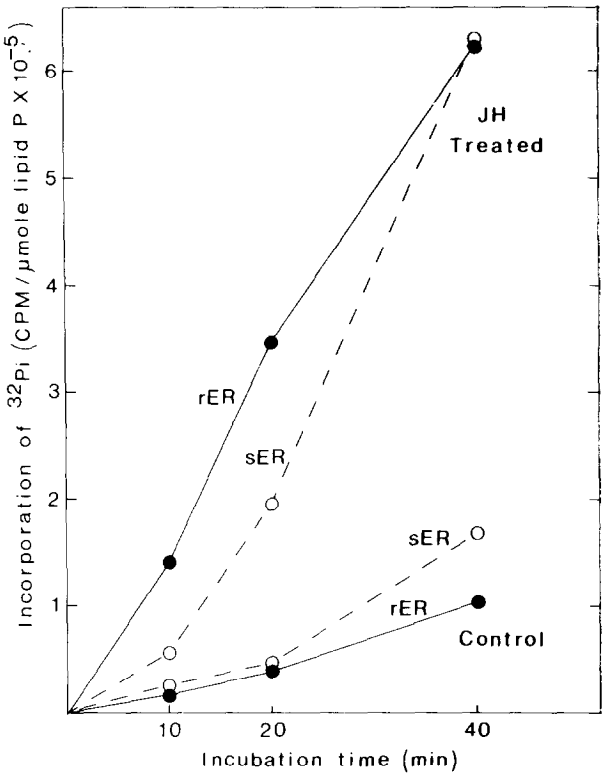

Figure II. Time-course of incorporation of [^{32}P]orthophosphate into fat body microsomal phospholipids during short-term in vitro organ culture. Four-week old CA$^-$ females were given 100μg of JH III (Calbiochem) dissolved in mineral oil by topical application (controls received mineral oil only). Three days later the fat bodies were removed and incubated individually in vitro in buffered saline (188mM NaCl, 1mM $CaCl_2$, and 19mM KCl made up in 50mM Tris-HCl, pH 7.5) containing 136μCi [^{32}P]orthophosphate. Following incubation with continuous shaking at 25°C for 10-40 minutes, the tissues were washed in ice-cold saline and immediately homogenized for microsome isolation. Each point represents the microsomal phospholipid obtained from the pooled fat bodies of four animals.

The incorporation of [^{32}P] into microsomal membranes, following JH treatment for three days in vivo, was determined during short-term incubation of fat body tissue in vitro. Under these conditions, the incorporation of label into PL of rough and smooth microsomes was nearly linear over a 40 minute period (Fig. II). The specific activity of PL from treated animals was about five-fold greater than that of the controls. We found that 70-80% of the radioactivity was contained within PTC.

Elevated rates of [^{32}P] incorporation into microsomes may reflect enhanced rates of isotope uptake by the cells rather than, or in addition to,

increased PL biosynthesis. Indeed, in vitro incubated cells from treated animals had a two-fold higher intracellular [^{32}Pi] specific activity than the controls (Table I). The total Pi pool per gram fat body was not affected, however, by hormone treatment (10.1 ± 0.5 (n=3) and 8.9 ± 0.3 (n=3) µmoles in JH-treated and controls respectively). On the other hand, no significant difference in intracellular [^{32}Pi] pools was observed after in vivo radiolabeling (Table I). Since even in vitro the magnitude of JH-enhanced labeling of microsomal PL was greater than the observed increase in precursor pool specific activity (Fig. II and Table I), we concluded that JH induced a net increase in the rate of membrane biosynthesis. The effect of JH was specific for fat body ER, since no increase occurred in the rate of labeling of sarcoplasmic reticulum PL from thoracic muscle (a non-target tissue) (Table I).

TABLE I. Changes in fat body intracellular [^{32}Pi] pools and incorporation of [^{32}P] into microsomal phospholipids after treatment of animals with 100µg JH III for three days.

	JH Treated (Spec. Act.)	Control (Spec. Act.)
In Vitro		
Intracellular [^{32}Pi] pool	245 ± 14	125 ± 19
Microsomal phospholipids	1.50 ± 0.18	0.43 ± 0.09
In Vivo		
Intracellular [^{32}Pi] pool	69.4 ± 7.3	54.5 ± 14.4
Microsomal phospholipids	3.86 ± 0.72	1.16 ± 0.10
Sarcoplasmic reticulum phospholipids	0.0437	0.0443

The in vitro incubations were carried out for 40 minutes under identical conditions as described in Figure II. The total microsome fraction was harvested and the TCA-soluble post-microsomal supernatant was used for determination of intracellular precursor-pool size. The pooled fat bodies from four CA$^-$ females were used for each replicate (n=3, mean ± standard error). Animals labeled in vivo (CA$^-$ females) were injected with 150 µCi of [^{32}P]orthophosphate for three hours and washed fat bodies were collected and fractionated as above. The pooled fat bodies from three animals were used for each replicate (n=3, mean ± standard error). Values for thoracic muscle sarcoplasmic reticulum represent the membrane lipids isolated from the pooled tissues of three animals.

The specific activity of the intracellular Pi pools is expressed as ^{32}Pi - CPM/µmole Pi x 10^{-5} and that of the phospholipids as ^{32}Pi - CPM/µmole lipid phosphorus x 10^{-5}.

The vitellogenin molecule of Leucophaea is a complex glycophospholipophosphoprotein (20). We isolated radiolabeled vitellogenin from microsomal vesicles (21) to determine the percentage of non-membrane PL radioactivity in the microsome fraction. We found that only 3% or less of the [^{14}C]choline counts, and less than 1% of the [^{32}P] counts, were due to radiolabeled vitellogenin lipid. We are therefore confident that radioactivity reported as microsomal PL represented primarily elements of the vesicle lipid-bilayer and not hormone-induced vitellogenin lipid contained within the vesicle lumen.

DISCUSSION

Hormones that induce the synthesis of exportable proteins are known to stimulate the rate of proliferation of rough and smooth ER in secretory cells (22,23). Since exportable proteins are synthesized primarily on membrane-bound polysomes (7,24,25), accelerated rates of protein synthesis may be coupled with the formation of new ER (23). Cells which produce vitellogenin are indeed rich in rough-surfaced ER (10-13,26). We now present biochemical evidence showing that vitellogenin synthesis and enhanced rates of ER proliferation, in an insect, are temporally coupled and induced by JH.

The formation of new ER directly parallels the JH-induced production of vitellogenin (Fig.I). By day 5 after JH administration--when most of the hormone has presumably been utilized--the rate of labeling of the ER returns to control levels (Fig. I). Vitellogenin production is similarly much reduced on day 5 (19) and virtually abolished by day 12 (Fig. I). We postulate that the rate of vitellogenin production is enhanced by the increased rate at which the ER is synthesized.

The increased rate of labeling of ER may represent an enhanced rate of synthesis and/or a decreased rate of degradation. Both of these components appear to contribute to the accumulation of smooth ER in phenobarbital-stimulated rat liver (27). We have no evidence whether the same is applicable for the effects of JH on fat body ER.

We find that JH enhances the rate of [^{32}Pi] uptake by fat body cells *in vitro*, resulting in intracellular pools that are two-fold higher than in the controls. However, no significant differences could be detected in intracellular [^{32}Pi] pools of cells labeled *in vivo* (Table I). Since the specific activity of microsomal PL in *in vitro*-incubated cells is 3.5-fold (Table I) to five-fold higher (Fig. II) in the hormone treated animals, the increased rates of [^{32}Pi] uptake cannot alone account for the enhanced rate of labeling of the membranes. We suspect that the observed changes in plasma membrane permeability to [^{32}Pi] may be artifacts of our *in vitro* incubation.

Exposure of adult females to JH results in a massive induction of vitellogenin synthesis, and a substantial increase in the rate of synthesis of other non-sex-specific hemolymph proteins (19, 28). During the peak response to JH, vitellogenin represents more than 80% of the total proteins synthesized and released by the fat body cells (21,28). Such a tremendous induction of exportable protein synthesis undoubtedly requires a synchronized proliferation of ER. The ER contains the terminal enzymes of phosphoglyceride biosynthesis (29,30) and is virtually the sole site of PL biosynthesis in eukaryotic cells (31,32). Since membrane constituents synthesized in the ER are physically transported to the cell surface during the process of protein secretion (32), the turnover rate for the membrane may parallel the rate of protein export. We suggest that the increased rate of production of membranes not only makes new binding sites available for vitellogenin polysomes, but also accelerates the rate of transport of ER-derived secretory vesicles to the cell surface. These events, all tightly coordinated by the chronic exposure of fat body cells to a single developmental hormone, namely JH, allow for the maintenance of prolonged periods of vitellogenesis that are necessary for reproduction.

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