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Transfer of cholesterol and diacylglycerol from lipophorin to *Bombyx mori* ovarioles in vitro: role of the lipid transfer particle

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Abstract

The objective of this study was to characterize the transfer of diacylglycerol (DAG) and cholesterol from larval *Bombyx mori* lipophorin to ovarioles. Transfer studies were carried out by incubating pupal ovarioles (5-day) with [³H]-cholesterol and [³H]-DAG-labeled lipophorin under different conditions. Transfer of both cholesterol and DAG exhibited hyperbolic dependency on lipophorin concentration with apparent K_m values of 0.83 ± 0.17 mg/ml and 0.74 ± 0.16 mg/ml, respectively. Pretreatment of ovarioles with anti-lipid transfer particle (LTP) IgG significantly inhibited transfer of labeled DAG to ovarioles (75%) and not cholesterol. Injection of *B. mori* pupae (day 4) with anti-LTP IgG significantly affected the weight (65%), number of eggs (49%), amount of lipid (74%), and protein (65%) of the adult ovaries. Matured eggs had a very faint yellow color and deformed shape compared to controls. The inhibitory effect demonstrates the active role LTP plays in growth of ovaries, development, and oogenesis. The effect on vitellogenin shortage on egg development and maturation was determined by implanting ovaries in male recipients that lack vitellogenin. An 80% decline in egg production was observed. However, the mature eggs were normal in shape, color, and lipid content. Thus, restricting lipid or protein delivery to developing ovaries would dramatically affect choriogenesis. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Cholesterol; Diacylglycerols; Lipid transfer particle; *Bombyx mori*; Oocytes

1. Introduction

Insect high-density lipophorin, HDLp, contains a wide variety of lipids, the majority of which constitute diacylglycerol (DAG). Lipophorin functions primarily as a reusable shuttle that delivers lipid from sites of absorption and storage to sites of utilization without being internalized at either site (Kawooya and Law, 1988). The majority of lipid delivery (about 90%) to developing oocytes in *M. sexta* takes place by the shuttling activity of the adult lipophorin, low-density lipophorin (LDLp) (reviewed by Canavoso et al. (2001). Another minor pathway for lipid delivery to oocytes involves endo-

cytosis of LDLp (5%) and its concomitant remodeling to HDLp (Kawooya and Law, 1988). A buffer-soluble lipoprotein lipase that in vitro hydrolyzes the DAG moiety of LDLp into glycerol and free fatty acids, which in turn are used for triacylglycerol (TAG) synthesis, has been identified in the yolk bodies of *M. sexta* eggs (Van Antwerpen and Law, 1992). In addition to lipid delivery by lipophorin, oocytes acquire about 5% of their lipid content from the female-specific yolk protein, vitellogenin. Vitellins, derived from vitellogenin are the primary source of amino acids for the developing ovaries (Osir et al., 1986).

In addition to the transport of DAG, lipophorin transports cholesterol from the midgut to fat body and ovaries (Yun et al., 2002; Jouni et al., 2002b). Cholesterol is the major sterol found in insects and serves as a structural component of cell membranes and as the precursor of the insect molting hormones, ecdysteroids (Grieneisen, 1994; Svoboda, 1999). In mature eggs of *M. sexta*, cholesterol is stored equally in the free and esterified forms

Abbreviations: Triacylglycerol (TAG); Diacylglycerol (DAG); Lipid transfer particle (LTP); 4; 2-aminoethylbenzene sulfonyl-fluoride (AEBSF); High-density lipophorin (HDLp); Low-density lipophorin (LDLp)

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(Jouni et al., 2002b). The exact mechanisms governing the unloading of DAG and cholesterol from lipophorin to oocytes and the contribution of a lipid transfer particle (LTP) are unknown.

LTP has been isolated from the hemolymph of several species (Canavoso et al., 2001) including *B. mori* (Tsuchida et al., 1997). The protein is composed of three subunits: apoLTP-I (350 kDa), -II (85 kDa) and -III (60 kDa). In vitro, LTP facilitates DAG transport from adult *M. sexta* fat body to lipophorin (Van Heusden and Law, 1989), from larval midgut to lipophorin (Canavoso et al., 2001), and the bi-directional transfer of DAG between larval fat body and lipophorin (Canavoso et al., 2002). Although available data clearly demonstrate the essential role LTP plays in promoting DAG transfer from cells to lipophorin, the mechanism is still unknown. One possibility is that LTP plays a role in the formation of a lipophorin–receptor complex. Alternatively, LTP might act to enhance the function of the tissue-specific lipid transfer factors, which could determine the direction of lipid transfer (reviewed by Arrese et al., 2001). We have recently shown that LTP does not facilitate the transfer of cholesterol from *M. sexta* larval midgut to lipophorin, from lipophorin to fat body (Yun et al., 2002) and from larval fat body to lipophorin (Jouni et al., 2002a).

In the present study, we characterized the transfer of cholesterol and DAG from lipophorin to *B. mori* ovarioles. We also demonstrated that LTP assists in the transfer of DAG, in vivo and in vitro, from larval lipophorin to ovarioles. But this protein plays no role in cholesterol transfer.

2. Materials and methods

2.1. Materials

[9,10-³H]-oleic acid was purchased from NEN (Boston, MA). (1,2n)-[³H]-cholesterol (sp. act. = 52 Ci/mmol) was purchased from Amersham Pharmacia Inc. (Piscataway, NJ). AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride], glutathione, dithiothreitol, benzamidine hydrochloride and DEAE-Trisacryl M were from Sigma (St. Louis, MO). Sodium [¹²⁵I]-iodide (17.4 Ci/mg) was purchased from DuPont-New England Nuclear (Wilmington, DE). IODO-GEN was from Pierce (Rockford, IL). Falcon multi-well tissue culture plates and cell strainers were obtained from Becton Dickinson (Franklin Lakes, NJ). Affi-Gel Protein A was from Bio-Rad (Hercules, CA). Centriprep Centrifugal Filter Devices were from Millipore–Amicon (Bedford, MA). Econo Pac DG columns were obtained from Bio-Rad. All other chemicals were analytical grade.

2.2. Insects

B. mori larvae of the N4 strain were reared on an artificial diet (Yakult, Japan) at 25 °C on a 12 h light/12 h dark photoperiod (Jouni and Wells, 1996). At the pupa stage, the insects were reared at 15 °C.

2.3. LTP purification

Larval *B. mori* hemolymph was collected into an ice-cold bleeding solution of PBS buffer (20 mM sodium phosphate of pH 6.5) containing 150 mM NaCl, and 5 mM EDTA, 1 mM glutathione, and 1 mM 4,2-aminoethylbenzene sulfonyl-fluoride (AEBSF), a serine protease inhibitor. The hemolymph was centrifuged at 3,000 g for 5 min to remove hemocytes and then subjected to two steps of KBr gradient ultracentrifugation as described previously (Shapiro et al., 1984; Prasad et al., 1986). After the first centrifugation at 5 °C and 50,000 rpm for 4 h in VTi50 rotor, lipophorin and LTP formed two yellow bands, with the LTP fraction having a higher density. Fractions containing LTP were subjected to a second ultracentrifugation for 16 h, as described above, except that the overlaying solution was a 1.2 g/ml KBr solution (Tsuchida et al., 1997).

LTP was collected from the top 4 ml of the tube, applied to an Econo Pac DG column and eluted with 40 mM sodium phosphate buffer, pH 6.0, containing 5 mM EDTA. The yellow fraction of LTP was applied to a DEAE Trisacryl M IBF column in the same buffer and eluted with a linear NaCl gradient 0–300 mM. The yellow fractions were pooled and applied to a Sephacryl S-300 column in bleeding solution and eluted with the same buffer. Fractions containing pure LTP, as judged by SDS-PAGE, were pooled and used to raise anti-LTP antibody. Specificity of anti-LTP IgG was determined by immunoblotting.

2.4. Isolation of [³H]-cholesterol- and [³H]-DAG-labeled lipophorin

Second and third day fifth instar larvae were fasted for 10 min and then fed a small piece of diet containing 4 μCi [³H]-cholesterol. Following consumption of the diet, the insects were placed on an unlabeled diet. Twenty-four hours later, cholesterol-labeled, high-density lipoproteins were isolated from the hemolymph by density gradient ultracentrifugation as previously described (Jouni et al., 2002b). Fractions containing lipophorin were dialyzed against 20 mM Tris-HCl buffer containing 150 mM NaCl, 2 mM EDTA, and 0.5 mM benzamidine hydrochloride (pH 6.5), concentrated using YM-100 centricon filters and washed with PBS buffer. Under these conditions, more than 95% of the labeled [³H]-cholesterol was present in lipophorin (Jouni et al., 2002b). The purity of the lipophorin preparations was

confirmed by SDS-PAGE analysis, using either a linear (7.5%) or gradient (4–15%) SDS-PAGE prepared as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250.

[³H]-DAG-labeled lipophorin was prepared following the same procedure described above, except that 2 μCi [³H]-oleic acid was fed to the larvae for 2 h prior to hemolymph collection. Under these conditions, more than 95% of the labeled [³H]-DAG was present in lipophorin (Jouni et al., 2000).

Pupal [³H]-cholesterol or [³H]-DAG lipophorin was prepared by injecting 2μCi of cholesterol or oleic acid respectively, into day-one pupae. This procedure was repeated for two consecutive days, hemolymph was collected from day-four pupae and used for the preparation of [³H]-labeled lipophorins following the same procedure described above. Preliminary data indicated that the rate of cholesterol and DAG incorporation into ovarioles was the same whether larval lipophorin or pupal lipophorin was used as the source of lipid, thus we used larval [³H]-labeled lipophorin in the subsequent experiments.

2.5. *In vitro* incubation of ovarioles with increasing concentrations of [³H]-cholesterol or [³H]-DAG lipophorin

Ovarioles from female pupae (5-day) were dissected on ice-cold PBS buffer containing 0.5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM glutathione, 0.1 mM diisopropylfluorophosphate, and 1 mM EDTA. To minimize variations between treatments, one ovary (four-ovarioles) was incubated in 300 μl Grace's medium containing increasing concentrations of [³H]-cholesterol-labeled lipophorin (0.1 μg/μl to 2 μg/μl, sp. act. = 400 cpm/μg), and the other ovary, obtained from the same pupa, was incubated in 300 μl Grace's medium containing [³H]-DAG-labeled lipophorin (0.1 μg/μl to 2 μg/μl, sp. act. = 433 cpm/μg). All incubations were carried out for 2 h at 25 °C on an orbital shaker. At the end of the incubation time, the tissues were separated from the incubation media by filtration using a cell strainer, washed thoroughly with PBS, and either frozen for further analysis or immediately lysed in 2 ml of 0.1 N NaOH and homogenized (Jouni et al., 2002a). Aliquots were used for lipid extraction (Folch et al., 1957). Organic layers were dried under a N₂ gas stream, resuspended in 500 μl chloroform and a sample used for radioactivity counting.

2.6. *Quantitation of lipophorin by radio-immunodiffusion*

Ovarioles from 5-day old pupae were incubated in 300 μl Grace's medium containing 200 μg [³H]-cholesterol-labeled lipophorin (sp. act. = 1400 cpm/μg), or [³H]-

DAG-labeled lipophorin (sp. act. = 428 cpm/μg). At the indicated time interval, the amount of radioactivity transferred to ovarioles was measured as mentioned above, and the concentration of lipophorin remaining in the incubation medium (15 μl) was determined by single radial immunodiffusion in 1% agarose gel containing 2% rabbit antiserum against fifth-instar lipophorin (Tsuchida et al., 1987). The components of the agarose gel were allowed to diffuse for 2 days at 4 °C.

2.7. *Effect of temperature on cholesterol and DAG transfer*

For these studies, 1 μg/μl of [³H]-cholesterol or [³H]-DAG lipophorin was used. Ovaries and labeled lipophorin(s) were pre-equilibrated at temperatures ranging from 4–42 °C prior to starting the transfer studies. Transfer studies were carried out as described above.

2.8. *Effect of anti-LTP IgG on cholesterol and DAG transfer to ovaries*

Antiserum against purified larval LTP was obtained from a New Zealand white rabbit as described by Canavoso et al. (2002). The IgG fraction was then purified using Affi-gel Protein A and stored at –80 °C. To elucidate the role of LTP in the transfer of cholesterol and DAG from lipophorin, ovaries were preincubated with non-immune rabbit IgG (control) or rabbit anti-LTP IgG (10 μg/μl Grace's medium). After 1 h the ovaries were washed with PBS buffer, transferred to 300 μl of Grace's medium containing 1 μg/μl of [³H]-cholesterol lipophorin (sp. act. = 400 cpm/μg) or [³H]-DAG-labeled lipophorin (sp. act. = 428 cpm/μg) and then transfer studies were carried for 2 h as described above.

2.9. *Role of LTP in the development of B. mori ovaries*

Ten pupae (4-day) were injected with 25 μl (600 μg) of anti-LTP IgG and the insects were then allowed to grow to adulthood at 15 °C. For control studies, ten pupae (4-day) were injected with 25 μl (600 μg) of non-immune rabbit IgG. After emergence, the ovaries were dissected out, the weight of the ovaries and number of eggs were recorded, and protein and lipid analyses were measured.

2.10. *Effect of vitellogenin on development of ovaries*

To determine whether the absence of vitellogenin — female-specific proteins — in growing ovaries is as detrimental as the lack of a lipid source, ovaries were implanted into males, following the procedure described by Takeda et al. (1996). The ovaries from pupae (0-day or 1-day) were dissected out under ice-cold Grace's

medium and all fat body tissues were removed. One ovary (four ovarioles) was implanted into a male pupa of the same age and the other ovary, obtained from the same female pupa, was implanted into a different female pupa (control). The recipients were kept on ice for 30 min before surgery after which a small cut was made between the intersegmental membranes where the implanted ovary was placed. After emergence, the ovaries were collected from female and male adults, homogenized in PBS buffer, and 25 μg samples analyzed on SDS-PAGE.

2.11. Lipophorin iodination and protein determination

Larval lipophorin (sp. act. 15845 cpm/ μg) was iodinated with sodium [^{125}I]-iodide using IODO-GEN following the manufacturer's instructions. To determine the extent of binding of lipophorin to ovarioles, 100 μg of [^{125}I]-labeled lipophorin was incubated with the ovarioles over a period of 4 h, and the amount of the radioactivity associated with the tissues (bound and internalized) was determined.

Protein concentrations were determined using a modified Lowry method with bovine serum albumin as a standard (Markwell et al., 1978).

Statistical tests were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). The results are expressed as percentages \pm SEM. $P < 0.05$ was considered as a significant difference between means.

3. Results and discussion

3.1. Time course of [^3H]-cholesterol and [^3H]-DAG lipophorin transfer studies

Fig. 1 shows the time course of cholesterol and DAG transfers when labeled lipophorin was incubated with pupal ovarioles. Time course data were best fitted on a one-exponential association curve ($r^2 = 0.99$ for both cholesterol and DAG plots). Over the 4 h incubation period, about 30% of the radioactive cholesterol was transferred to ovarioles, and was characterized by a $t_{1/2}$ of 3 h (Fig. 1A). In contrast, the majority (80%) of the radioactive DAG of lipophorin was transferred to ovarioles (Fig. 1B) and was characterized by a $t_{1/2} = 1$ h. The progressive and significant increase in the accumulation of cholesterol and DAG in ovarioles following incubation with lipophorin corresponded to the gradual loss of the lipid from lipophorin without internalization of the protein ($< 5\%$) as confirmed by immunodiffusion assays (Fig. 1). Similarly, when [^{125}I]-labeled lipophorin was incubated with ovarioles over a period of 4 h, the amount of the radioactivity associated with the tissues (bound and internalized) did not exceed 5% (Fig. 2).

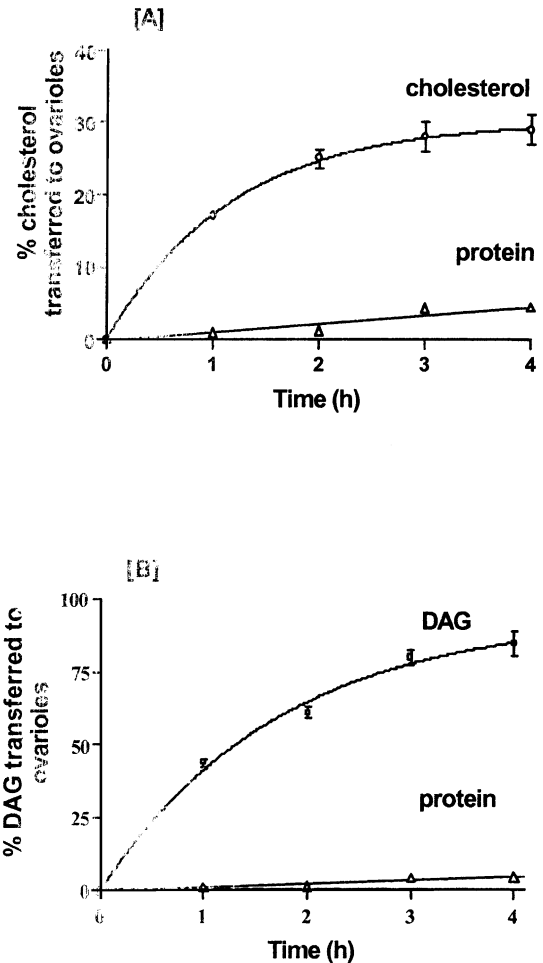


Fig. 1. The time course of cholesterol and DAG transfer to ovarioles. *B. mori* ovarioles were incubated in 300 μl Grace's medium containing 200 μg [^3H]-cholesterol-labeled lipophorin (sp. act. = 1400 cpm/ μg) [A] or [^3H]-DAG-labeled lipophorin (sp. act. = 428 cpm/ μg) [B]. At the indicated time intervals, the ovarioles were washed, homogenized and lipid contents were extracted using the Folch method. A sample of the extracted lipids was used to measure the radioactivity transferred to ovarioles. Concentration of the protein moiety of lipophorin remained in the incubation medium was determined by single radial immunodiffusion in 1% agarose gel containing 2% rabbit antiserum against fifth-instar lipophorin. Values represent percentages of five determinations \pm SEM (note the difference scales on the two graphs).

Kawooya and Law (1988) reported similar results where *M. sexta* follicles were able to uptake relatively small amounts of lipophorin early in the development. As the follicles increased in size, there was a corresponding increase in the endocytosis of lipophorin. However, this uptake was halted when the follicle reached 1.0 mm in size, and was attributed to the formation of occlusion zones in the follicular epithelium. Nevertheless, the endocytosis of lipophorin did not exceed 10%.

3.2. Effect of temperature on cholesterol and DAG transfer to ovarioles

Cholesterol and DAG transfers to ovarioles were strongly temperature dependent (Fig. 3). Transfer of

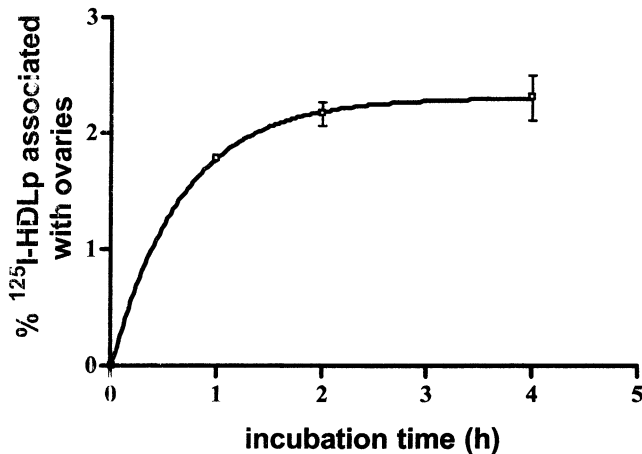


Fig. 2. Association of [¹²⁵I]-lipophorin with ovarioles. Eight ovarioles, obtained from one pupa, were incubated with 100 μg of [¹²⁵I]-lipophorin in 250 μl Grace's medium for 4 h at room temperature. At the indicated time intervals, the ovarioles were washed three times with PBS and the radioactivity was counted using a gamma counter. Values represent percentages of three determinations ± SEM.

cholesterol and DAG increased linearly with increasing temperature. This is in agreement with our recent report that the transfer of cholesterol from *M. sexta* fat body to lipophorin was temperature dependent (Jouni et al., 2002a). Using the Arrhenius plots, we calculated an activation energy for DAG transfer of 36.2 ± 8.3 kJ/mol and 56.6 ± 10.1 kJ/mol for cholesterol transfer. The mechanisms by which DAG and cholesterol are transferred to ovarioles are yet to be elucidated. However, the calculated activation energies support the predicted values for an aqueous diffusion mechanism for cholesterol transfer and for a receptor-mediated process for DAG transfer (Yancey et al., 1996).

3.3. Transfer of cholesterol and DAG from increasing concentration of lipophorin

The amount of labeled cholesterol transferred to pupal ovarioles (5-day) increased with increasing the concentrations of lipophorin (0.25 mg/ml to 2 mg/ml) (Fig. 4). Analysis of the transfer data by non-linear least squares demonstrated a saturation behavior with an apparent K_m value of 0.83 ± 0.17 mg/ml (Fig. 4A). Similarly, the amount of labeled DAG transferred to ovarioles showed a saturation behavior with an apparent K_m value of 0.74 ± 0.16 mg/ml (Fig. 4B). In contrast, a negligible amount of radioactivity was found in ovarioles when incubations were performed at 4 °C (data not shown).

The observed saturable process is consistent with two different mechanisms by which lipids (cholesterol and DAG) are transferred to ovarioles. The first mechanism is a receptor-mediated pathway. In vertebrates this pathway is characterized by low activation energy of about 10 kJ/mol (Yancey et al., 1996). Based on the observed low activation energy for DAG transfer to ovarioles

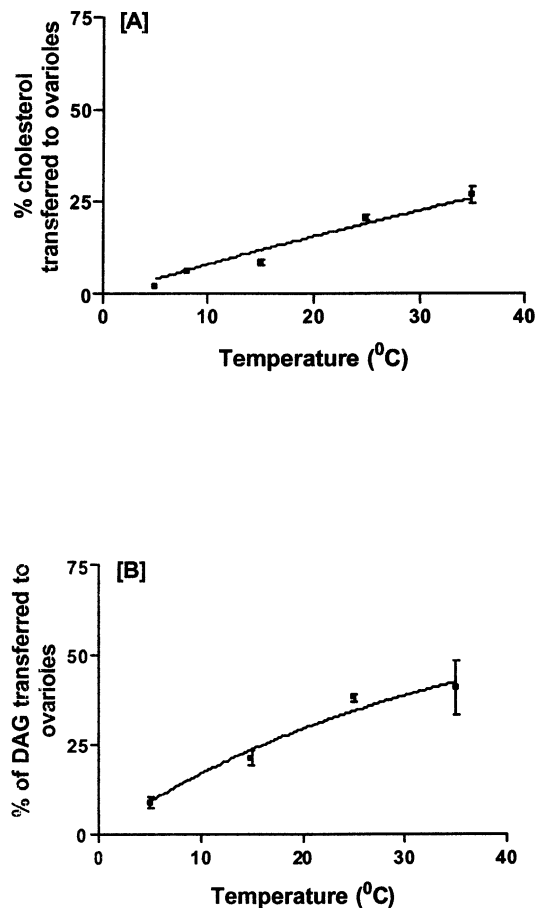


Fig. 3. Effect of temperature on radiolabeled cholesterol and DAG transferred to ovarioles. Ovaries obtained from 4-day pupae and [³H]-cholesterol lipophorin (sp. act. = 400 cpm/μg) or [³H]-DAG-labeled lipophorin (sp. act. = 428 cpm/μg) were pre-equilibrated to temperatures ranging from 4–42 °C prior to starting the transfer studies. [A] One ovary (4-ovarioles) was incubated in 300 μl Grace's medium containing [³H]-cholesterol-labeled lipophorin. [B] The second ovary, obtained from the same pupa, was incubated in 300 μl Grace's medium containing [³H]-DAG-labeled lipophorin. At the end of the incubation time (2 h), aliquots of the homogenized ovarioles (refer to Experimental Section for more details) were used for radioactivity counting. Values represent percentages ± SEM of three determinations.

(36.2 ± 8.3 kJ/mol) and our recent report that DAG is transferred from *M. sexta* lipophorin to fat body via a receptor-mediated process (Canavoso et al., 2002), it is likely that DAG transfer to ovarioles is also receptor dependent. In a receptor-mediated process lipophorin would bind its receptors on the ovarioles and deliver its DAG. Thus, the longer the incubation of lipophorin with the ovarioles, the more DAG is expected to be transferred. The second mechanism that accounts for the observed saturation is the spontaneous desorption of the lipids from lipophorin into the aqueous solution followed by a second-order interaction between the desorbed lipids and membranes (Davidson et al., 1995). In mammalian systems this mechanism is characterized by activation energy of about 75–100 kJ/mol (Yancey et al.,

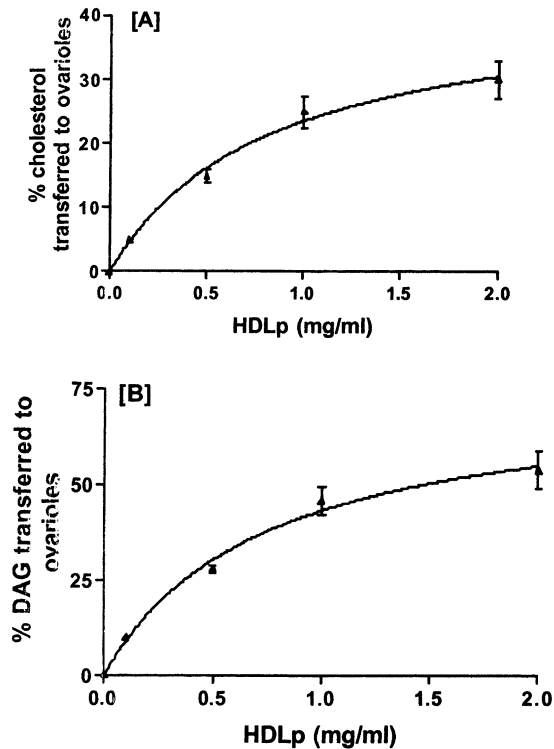


Fig. 4. Concentration dependence of cholesterol and DAG transfer. *B. mori* ovarioles were incubated with different concentrations of [^3H]-cholesterol-labeled or [^3H]-DAG-labeled lipophorin ranging from 0.25 mg/ml to 2 mg/ml. After 2 h, the ovarioles were washed, homogenized, and the amount of radioactivity transferred to the tissues was measured using a scintillation counter. [A] Percent of [^3H]-cholesterol transferred to ovarioles. [B] Percent of [^3H]-DAG lipophorin transferred to ovarioles. Values represent percentages of three determinations \pm SEM.

1996). Because the high activation energy of 56.6 ± 10.1 kJ/mol is more in line with that predicted for an aqueous diffusion mechanism than that predicted for a receptor-mediated process, and the fact that an aqueous diffusion mechanism occurs for cholesterol transfer from *M. sexta* fat body (Jouni et al., 2002a) or midgut and from lipophorin to fat body (Yun et al., 2002) suggest that cholesterol transfer to ovarioles could also take place by a simple aqueous diffusion, where the sterol is transferred from donor to acceptor following its concentration gradient.

3.4. Role of LTP in cholesterol and DAG transfer from lipophorin to ovarioles

LTP is a very high-density lipoprotein isolated from the hemolymph of several species (reviewed by Arrese et al. (2001)). The protein is characterized by its ability to facilitate transport of DAG from adult *M. sexta* fat body to lipophorin in vitro (Van Heusden and Law, 1989), from midgut of larval *M. sexta* to lipophorin (Canavoso and Wells, 2001), and the bi-directional transfer of DAG between fat body and lipophorin (Canavoso et al., 2002).

Treatment of ovarioles with anti-LTP IgG before incubation with cholesterol-labeled lipophorin exhibited no effect on the transfer of cholesterol to ovarioles, indicating that LTP does not facilitate this transfer process (Fig. 5A). In contrast, treatment of ovarioles with anti-LTP IgG prior to incubation with DAG-labeled lipophorin resulted in a significant inhibition (75%) of labeled-DAG transfer to ovarioles (Fig. 5B). No inhibition of [^3H]-DAG transfer from lipophorin to ovarioles was observed when the tissue was preincubated with non-immune rabbit serum IgG, demonstrating that the inhibitory effect of anti-LTP antibody was specific.

Ovarioles contain intrinsic LTP as the transfer of DAG from lipophorin to ovarioles occurred in the absence of added LTP (Fig. 5B). In *M. sexta*, the protein is synthesized by the fat body and secreted into the hemolymph (Van Heusden et al., 1996). Whether ovarioles synthesize LTP is yet to be determined. It is likely that binding of hemolymph-LTP to ovarioles, which cannot be eliminated even with extensive washings, is sufficient to facilitate lipid transfer. Association of LTP with *M.*

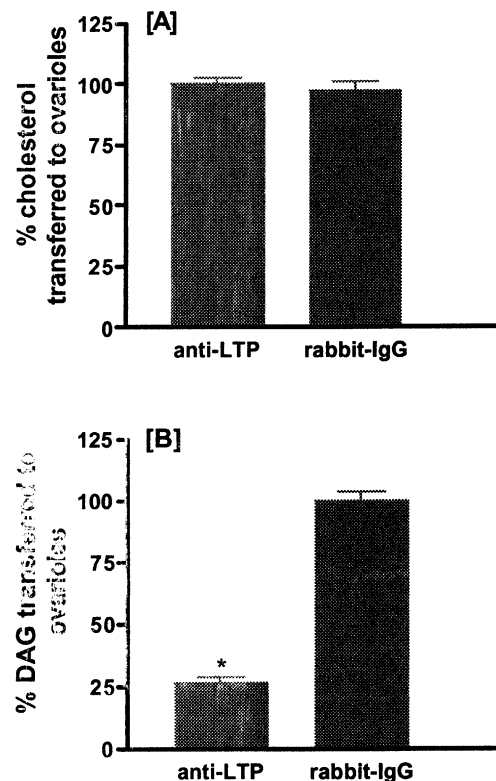


Fig. 5. Effect of Anti-LTP IgG on cholesterol and DAG transfer to ovarioles: To elucidate the role of LTP in the transfer of cholesterol and DAG from lipophorin, ovaries were preincubated with non-immune rabbit IgG (control) or anti-LTP IgG ($10 \mu\text{g}/\mu\text{l}$ Grace's medium). After 1 h the ovaries were extensively washed with PBS buffer, and then transferred to Grace's medium containing [^3H]-cholesterol-labeled lipophorin [A] or [^3H]-DAG-labeled lipophorin [B] and transfer studies were carried out for 2 h as described in the methodology section. Values represent percentages \pm SEM of four determinations.

Table 1
Effect of anti-LTP injection on development of ovaries

Treatment	Ovary weight (mg)	Number of eggs	Amount of lipid (μg)	Amount of protein (μg)
Anti-LTP	93 \pm 23	191 \pm 16	2724 \pm 292	5451 \pm 810
Serum IgG	264 \pm 29	373 \pm 16	10401 \pm 875	15428 \pm 1630

B. mori pupae (4-day) were injected with 600 μg of anti-LTP IgG. For control studies, another set of pupae, of the same stage, were injected with non-immune rabbit IgG. After emergence (1-day) the ovaries were dissected out and their weight was recorded. Number of eggs was counted and protein and lipid analyses were determined. Values represent averages \pm standard deviations for 10 determinations. Significantly different at $p < 0.001$.

sexta midgut that does not synthesize the protein was also reported (Canavoso and Wells, 2001).

3.5. Role of LTP in import of lipid during ovary development in vivo

Injection of *B. mori* pupae (4-day) with anti-LTP IgG caused a significant inhibitory effect on the weight (65%), number of eggs (49%), amount of lipid (74%) and protein (65%) of the adult ovaries compared to control insects that were injected with non-immune rabbit serum IgG (Table 1). The eggs obtained from adults that were injected with anti-LTP IgG during the pupal stage were deformed in shape compared to controls (Fig. 6). The deformed eggs were characterized by a thinly elongated shape and a faint yellow color compared to the oval shape and bright yellow color of control eggs. At the pupal stage the insects are not feeding while the ovarioles are developing and increasing in size and in lipid and protein content. The developing ovaries acquire their lipid by mobilizing DAG from fat body via the hemolymph proteins, lipophorin and vitellogenin. In *M. sexta* the contribution of lipophorin-mediated lipid delivery (90%) to ovaries is far greater than vitellogenin con-

tribution (5%) (Kawooya and Law, 1988). It is worth mentioning that ovaries do not have the machinery for the de novo synthesis of TAG (Van Antwerpen et al., 1998), thus necessitating lipid delivery by lipophorin. Even if de novo synthesis of TAG exists, it is not sufficient to sustain growth and maturation of the eggs. What is clear from the in vivo injection of anti-LTP IgG into the pupae is the hindering of two lipid transfer processes; namely i) the transfer of DAG from fat body to lipophorin and ii) lipophorin–DAG uptake by the ovaries. This is supported by the fact that the transfer of DAG was significantly decreased when adult *M. sexta* fat body was treated with anti-LTP IgG in vitro (Van Heusden and Law, 1989), and when *B. mori* ovaries were treated with anti-LTP IgG (Fig. 5B). The inhibitory effect reveals the active role LTP plays in lipid mobilization to ovary and thus in oogenesis. At this time we do not know whether LTP facilitates the transfer of DAG from vitellogenin to ovaries.

3.6. Vitellogenin is required for ovary maturation

In *B. mori*, yolk granules are composed of three major proteins, vitellin, a 30K protein, and an egg-specific protein (Takeda et al., 1996). The egg-specific protein is produced in the follicle cells (Sato and Yamashita, 1991). Vitellogenin and the 30K protein are synthesized in the fat body, secreted into the hemolymph, and sequestered by the developing ovaries. Vitellins, derived from vitellogenin, the female-specific protein, are the major egg protein constituents in insects and the most important source of amino acid for the developing embryo (Osir et al., 1986).

To determine if a deficiency in vitellogenin would have the same detrimental effect on development of ovary and egg maturation as observed for lipid deficiency, ovaries were implanted into male pupae and their maturation and development were examined. SDS-PAGE analyses of the pupal hemolymph and adult ovarian proteins are shown in Fig. 7. As expected, only hemolymph obtained from female pupae contained vitellogenin (Fig. 7, Lane 1). Male-implanted ovaries contained minute amounts of vitellins whereas ovaries implanted in females had substantial amounts of vitellins

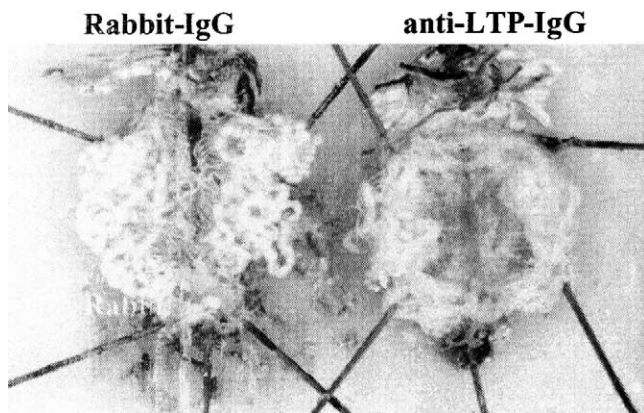


Fig. 6. Role of LTP on the development of ovaries. [A]: 4-Day *B. mori* pupae were injected with 600 μg of anti-LTP IgG and the insects were grown to adulthood at 15 °C. [B]: For control experiments, *B. mori* pupae (4-day) were injected with non-immune rabbit IgG. After emergence, the adults were dissected and pinned down with dissecting pins and photographed.

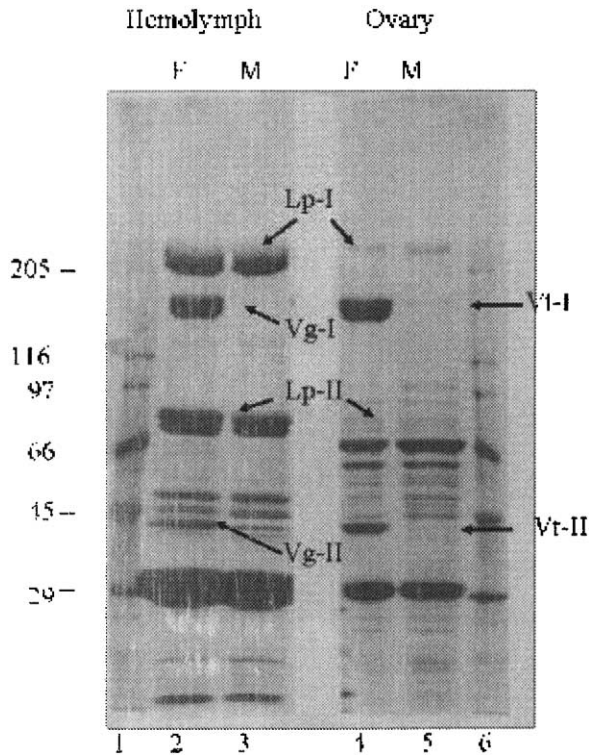


Fig. 7. SDS-PAGE analysis of the pupal hemolymph and adult ovarian proteins. One ovary (4-ovarioles) obtained from a pupa (0-day) was implanted into a male pupa of the same age and the pair of ovaries obtained from the same pupa was implanted in another female pupa. After emergence, the ovaries were collected from female and male adults, homogenized in PBS buffer and 25 μ g samples were analyzed on SDS-PAGE. Protein profile of the pupal female hemolymph (lane 2), pupal male hemolymph (lane 3) and ovaries obtained from adult-implanted female (lane 4) and from adult-implanted male (lane 5). Lanes 1 and 6 are molecular weight markers. Lp (lipophorin), Vg (vitellogenin), Vt (vitellin).

(Fig. 7, Lane 4). Ovaries implanted in males grew larger in size compared to when they were first implanted, but were considerably smaller than female-implanted ovaries (control). As a result, few eggs (30 per ovary) were made by male-implanted ovaries, representing an 80% decline in egg production (data not shown). Surprisingly, these eggs had normal shape, color, and lipid content. The fact that male-implanted ovaries made very few eggs with normal lipid content emphasizes the primary function of lipophorin, i.e., selective delivery of lipid rather than a source of amino acid during embryogenesis. The ability of male *B. mori* to grow and produce several eggs is attributed to the uptake of vitellogenin by ovaries prior to implantation and the continual uptake of lipid from lipophorin after implantation. It is known that in insects the major causes of oocyte resorption — production of fewer eggs — are starvation (Bell and Bohm, 1975) and shortage of vitellogenin (Shalom et al., 1988). Thus, it is conceivable to assume that the shortage of vitellogenin in male-implanted ovaries was a major determinant of egg production. Therefore, restricting lipid or protein

delivery to developing ovaries would stunt growth and dramatically affect choriogenesis.

4. Summary and Conclusions

We have demonstrated that a deficiency in vitellogenin by implanting ovaries in males or the interference of DAG transport to ovaries by injecting anti-LTP IgG drastically affected egg growth and maturation. Both cholesterol and DAG are transported to ovaries by lipophorin. Cholesterol and DAG demonstrated a saturation behavior for the amount of lipophorin used.

We propose that cholesterol and DAG are transported to ovarioles by an aqueous diffusion and a receptor-mediated mechanisms, respectively. The aqueous diffusion mechanism for cholesterol transfer to the ovary does not require lipophorin binding to the ovarian membrane receptors, whereas DAG delivery to ovarioles takes place by two different pathways: a) A minor pathway that requires the binding of lipophorin to its receptor prior to being endocytosed. Once inside the ovarioles, a lipoprotein lipase hydrolyzes DAG into fatty acids and glycerol, which in turn are used for the synthesis of TAG. This is supported by the fact that a membrane-bound lipoprotein lipase has been characterized at the surface of the *M. sexta* oocytes, which in vitro hydrolyzes DAG into free fatty acids and glycerols (Van Antwerpen et al., 1998). b) The second pathway for DAG delivery to ovarioles is the selective transfer from lipophorin. This pathway requires the binding of lipophorin to its receptor and the participation of LTP. We demonstrated that LTP plays a critical role in facilitating the transfer of DAG and not cholesterol from lipophorin to ovarioles. The findings of this report in conjunction with our recent publications reveal the physiological role LTP plays in lipid metabolism: DAG loading of lipophorin at the midgut, its transfer to the fat body for feeding larvae, and mobilization from fat body to ovaries. The functional diversity of lipophorin in insects is remarkable. What is intriguing about its function is the ability of lipophorin to deliver different lipids; cholesterol and DAG, to the same target tissues; fat body, midgut and to ovarioles, by two distinct mechanisms.

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