Developmental down-regulation of receptor-mediated endocytosis of an insect lipoprotein

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Abstract Fat body cells of insects exhibit a high-affinity lipoprotein binding site at their cell surfaces. In the present study, the lipoprotein binding site was identified as an endocytotic receptor involved in receptor-mediated uptake of its lipoprotein ligand, high density lipophorin. After an initial period of high endocytotic uptake of high density lipophorin in the adult stage, this process strongly diminished. In the same period, a dramatic increase in cell surface-associated lipoproteins was observed. When animals were starved, however, internalization of lipoproteins was maintained. The pathway followed by the internalized lipoproteins appears to be different from the endosomal/lysosomal pathway, as the vast majority of apolipoproteins seemed to escape from lysosomal hydrolysis. In addition, no substantial intracellular accumulation of apolipoproteins was observed, suggesting that internalized lipoproteins were resecreted. It is unlikely that internalization is required for transport of the two major lipid components of insect lipoproteins, diacylglycerol and cholesterol, as inhibition of endocytosis neither affected the exchange of these lipids between lipoproteins and fat body cells nor influenced the loading of diacylglycerol onto lipoproteins in response to adipokinetic hormone. We postulate that the endosomal environment may facilitate transport of components which, unlike diacylglycerol and cholesterol, cannot be transported by simple aqueous diffusion.—**Dantuma, N. P., M. A. P. Pijnenburg, J. H. B. Diederen, and D. J. Van der Horst.** Developmental down-regulation of receptor-mediated endocytosis of an insect lipoprotein. *J. Lipid Res.* 1997. **38:** 254–265.

Supplementary key words retroendocytosis • lipophorin • fat body • locust • adipokinetic hormone • lipid mobilization • lipid storage

An important physiological phenomenon is the ability of some cells to selectively exchange lipids with lipoproteins bound to the cell surface. Selective lipid transport has been reported for different cell types and appears to be common in nature (1–4).

The majority of the circulating lipids in insects is transported by a selective mechanism, which renders insects an appropriate model to study selective lipid transport between lipoproteins and target cells (5). Catabolic lipids in insects are transported as diacylglycerol (DAG) by lipophorin, an abundant lipoprotein in the hemolymph. High density lipophorin (HDLp), which contains two non-exchangeable apolipoproteins, apolipophorin (apoLp)-I and -II (6), delivers DAG to the recipient cells, such as the fat body cells, without degradation of the lipoprotein matrix (2, 4, 7). As a result, the lipoproteins can be discharged and reloaded and in this way function as reusable lipid shuttles. This mechanism enables insects to transport large amounts of DAG within a short time period. Lipid mobilization is pertinent especially during sustained flight activity of migratory insects (4, 7), when HDLp is loaded at the fat body to the larger molecular mass low density lipophorin (LDLp) by adding DAG derived from the lipid stores in the fat body cells and several molecules of an exchangeable apolipoprotein present in the hemolymph, apoLp-III, in response to adipokinetic hormones (AKHs) (8). Thus, fat body cells are not only able to selectively extract DAG from HDLp but they can also load HDLp with DAG cargo.

Previously, a high-affinity HDLp binding site at the fat body of migratory locusts was characterized (9, 10). We have recently demonstrated that the number of cell surface-localized binding sites increases during the adult stage whereas the total number of binding sites appears to remain constant (11). Based on these observations a model was postulated that implies that a part of the HDLp binding sites is present on intracellular membranes, whereas afterwards, ca. day 7 after the imaginal ecdysis, a redistribution of binding sites occurs from an intracellular pool to the plasma membrane.

The results reported in the present study are consistent with this model. We demonstrate that the HDLp

Abbreviations: AKH, adipokinetic hormone; apoLp, apolipophorin; DAG, diacylglycerol; DiI, 1, 1′ dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; HDLp, high density lipophorin; LDLp, low density lipophorin.

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binding site is an endocytotic lipoprotein receptor and that a developmental down-regulation of endocytotic uptake of HDLp coincides with an increase of binding of HDLp to the cell surface.

MATERIALS AND METHODS

Animals

Locusts, *Locusta migratoria,* were reared under crowded conditions as described previously (12). All experiments except isolation of unlabeled lipoproteins were performed with male locusts.

Isolation and labeling of lipoproteins

Hemolymph was collected from adult locusts (15–20 days after the imaginal ecdysis) and used for the isolation of HDLp by subjecting pooled samples to density gradient ultracentrifugation (11).

The lipoprotein lipid moiety was fluorescently labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) according to Pitas et al. (13) with slight modifications of the original method. Labeling incubations were performed in HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, 10 mM KCl, pH 7.0) in the presence of 0.02% sodium azide. Free DiI was separated from DiI-labeled lipoproteins using a PD10 column (Pharmacia). Only DiI incorporated in the lipoproteins enters the column matrix whereas free DiI sticks at the top of the column. Fluorescein-succinimidyl ester (Molecular Probes) was used to label the lipoprotein protein moiety. Lipoproteins were incubated with a large molar excess of fluorescein succinimidyl ester (protein/fluorescein: 1/100) in 0.1 M boric acid (pH 8.5) for 1 h at 4°C. After incubation, free label was linked to glycine by a subsequent incubation for 5 min in the presence of a molar excess of glycine (glycine/fluorescein: 10/1). Labeled lipoproteins and fluorescein linked to glycine were separated by gel filtration.

Radiolabeling of the lipoproteins in the protein moiety was performed using N-succinimidyl [2,3-^{3}H]propionate (Amersham International) according to Bolton and Hunter (14). Lipoproteins containing tritiated DAG were isolated by density gradient ultracentrifugation of hemolymph collected from adult locusts that had been fed 2 μCi [9,10-^{3}H]triolein (DuPont, NEN Research Products) 2 h prior to collection. Lipoproteins labeled with tritiated cholesterol were obtained by isolation from hemolymph of locusts that had been injected 10 min prior to collection with 2 μCi [1,2-^{3}H]cholesterol (DuPont, NEN Research Products) per locust. Radioactivity in the hemolymph was mainly recovered in the lipoprotein fraction with both labeling methods. Thin-layer chromatography of the lipid moiety of the lipoproteins labeled by triolein feeding revealed that more than 90% was present in the 1,2-DAG fraction.

Fluorescence microscopy

Fat body tissue from young and older adults, 4 and 15 days after the imaginal ecdysis, respectively, and from starved older adults was dissected and rinsed in medium A (HEPES-buffered saline +4 mM $CaCl_2$ +2 mM $MgCl_2$). The starved adults had been reared without food but with water-rinsed cotton-wool from day 8 to day 14 after the imaginal ecdysis and then had been fed a normal diet on day 15, one day before killing the animals. The tissue was incubated with fluorescently labeled HDLp in medium A for 90 min at 30°C. After incubation, the tissue was rinsed 3 × 10 min in 0.1 M phosphate buffer (pH 7.4) at 4°C and fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 16 h at 4°C. Fixed tissue was embedded in agar and 20-μm vibratome slices were cut, which were studied using a fluorescence microscope (Axioskop, Zeiss) with an appropriate filter setting. Effects of inhibition of luminal acidification on endocytosis were investigated using 10 mM NH_4Cl or 100 μM chloroquine (Sigma) (15). The endocytotic compartment in fat body cells was labeled by incubating the tissue with 10 mg/ml of the fluid-phase endocytosis marker Cascade Blue hydrazide (Molecular Probes).

Lipid and apolipoprotein cell association and degradation

DAG, cholesterol, and apolipoprotein uptake were measured by incubating fat body tissue in medium A at 30°C with HDLp that was radioactively labeled in the DAG, cholesterol, or protein moiety, respectively. Aliquots of the incubation medium were taken at several time points and radioactivity was determined. The decrease in the amount of a certain radioactive label in the medium was considered to be equal to the amount of cell-associated label. Cell association of the different labels is expressed as equivalents of HDLp protein, which indicates the amount of lipoproteins expressed as HDLp protein, that contained the indicated amount of label at the start of the incubation.

Degradation of HDLp was quantified by incubating fat body tissue with HDLp tritiated in the protein moiety for 6 h at 30°C and rinsing 2 × 5 min in 5 ml medium A. After incubation the tissue was homogenized in distilled water by sonification. The incubation medium and an aliquot of the tissue homogenate were precipitated with 10% trichloroacetic acid for 30 min at 4°C and centrifuged for 10 min at 14,000 *g*. The acid-soluble fraction present in a control incubation without fat body tissue

was subtracted from the amount of acid-soluble label present in the incubation medium. Acid-soluble label was regarded as degraded apolipoproteins (16). A second aliquot of the tissue homogenate was counted in order to determine the total amount of cell-associated label. When indicated, experiments were performed in the presence of 10 mM NH_4Cl or 100 μM chloroquine.

The amount of fat body tissue used in the incubations was quantified by determining total protein content after a chloroform–methanol extraction as described previously (11) using the protein determination method of Schacterle and Pollack (17).

In vitro lipid mobilization

In vitro LDLp formation was accomplished using fat bodies of adult locusts, 15 days after the imaginal ecdysis, as described elsewhere (18). For each experiment a pool of 4 fat bodies was used. Each individual fat body was incubated with 1 mg HDLp in the presence or absence of 10 pmol AKH-I (Peninsula Laboratories). The lipoproteins were separated after the incubation by density gradient ultracentrifugation (11) and A_{280} was monitored during fractionation.

Statistical analysis

Differences between groups were tested with a Student's *t*-test. Paired Student's *t*-tests were used when the data of the different groups had been obtained with fat body tissue samples of the same individuals. Otherwise unpaired Student's *t*-tests were applied.

Data are expressed as mean ± standard error of the mean (SEM).

RESULTS

Endocytotic internalization of HDLp

In insects, the fat body is surrounded by the hemolymph from which it recruits the lipoproteins (19). Consequently, incubation of intact fat body tissue in lipoprotein-containing medium as applied in this study mimics the in vivo situation.

Initially, fat body tissue of young adults, 4 days after the imaginal ecdysis, was used to study internalization of fluorescently labeled HDLp. We expected that if endocytosis of HDLp occurs, this would be optimal at this developmental stage in view of the postulated redistribution of HDLp binding sites around day 7 after the imaginal ecdysis.

Incubation of fat body tissue of young adults with DiI-labeled HDLp resulted in a punctated fluorescent staining throughout the cytoplasm (**Fig. 1A**; for comparison a semi-thin section of control fat body is shown in Fig. 1F). This punctated staining was observed even at very low DiI-HDLp concentrations (0.01 mg/ml). At higher concentrations of DiI-HDLp (0.5–1.0 mg/ml), which are still far below the physiological concentrations of HDLp in the hemolymph (8.5 and 17.7 mg/ml in young and mature adult locusts, respectively (20)), DiI-HDLp was also observed at the superficial plasma membranes, which directly contact the hemolymph, and in the intercellular spaces (not shown).

As such a punctated intracellular staining is indicative of endocytotic uptake, we examined more closely the possibility of receptor-mediated uptake of HDLp. Receptor-mediated endocytosis of a ligand is known to be saturable and, consequently, endocytotic uptake of the labeled ligand can be inhibited by adding an excess of unlabeled ligand. An excess of unlabeled HDLp strongly reduced the punctated intracellular staining of DiI-HDLp (Fig. 1B), implying internalization of HDLp to be facilitated by specific receptors.

Two agents, ammonium chloride and chloroquine, that inhibit luminal acidification of the endosomal/lysosomal compartment (15), and which are known to affect internalization and trafficking of ligands that are subject to receptor-mediated endocytosis (21), were tested. Both ammonium chloride (Fig. 1C) and chloroquine (Fig. 1D) reduced the amount of intracellularly localized DiI-HDLp at low DiI-HDLp concentrations. However, no differences between incubations with and without chloroquine were observed at higher DiI-HDLp concentrations (0.2–0.5 mg/ml), whereas ammonium chloride reduced the punctated staining also at these higher concentrations almost completely (not shown).

In order to verify whether the punctated staining observed with HDLp labeled with the fluorescent lipid DiI corresponds with the localization of lipoprotein particles, fat body tissue was incubated with HDLp labeled with fluorescein in the protein moiety. This resulted in a similar staining pattern in the cytoplasm and intercellular spaces as observed with DiI-HDLp (Fig. 1E). With fluorescein-HDLp the intracellular punctated staining was most clearly visible when fat body tissue was incubated with 0.1 mg/ml fluorescein-HDLp. However, incubation with 0.01 mg/ml fluorescein-HDLp also resulted in a similar fluorescent staining pattern. Thus, not only the lipid moiety of the lipoprotein is subject to endocytotic uptake, but the entire lipoprotein appears to be internalized.

In addition, we demonstrated that the intracellular DiI-HDLp is present in the endocytotic compartment of fat body cells. Simultaneous incubation of fat body cells for 90 min with 0.01 mg/ml DiI-HDLp and 10 mg/ml of the fluorescent fluid-phase endocytosis marker

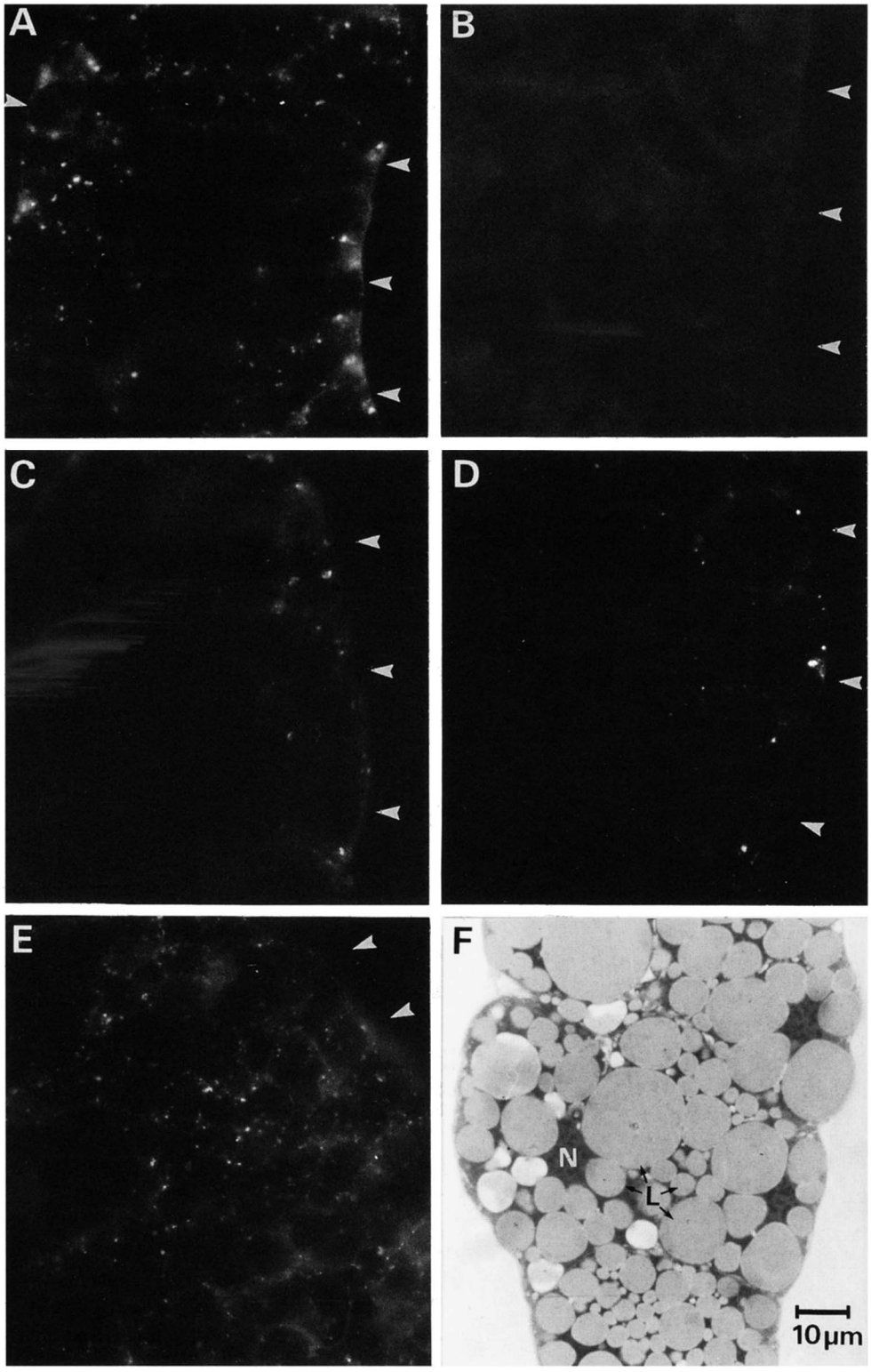

Fig. 1. Internalization of HDLp by fat body tissue of young adults. Fat body tissue of young adult, 4 days after imaginal ecdysis, was incubated for 90 min at 30°C with 0.01 mg/ml HDLp that had been labeled with the fluorescent lipid DiI, without additives (A), in the presence of an excess of unlabeled HDLp (5 mg/ml) (B), in the presence of 10 mM NH_4Cl (C), and in the presence of 100 µM chloroquine (D). Fat body tissue of young adult was incubated similarly with 0.1 mg/ml HDLp that had been labeled in the protein moiety with fluorescein (E). Vibratome sections (20 µm) of agar-embedded tissue were examined with a fluorescence microscope. A methylene blue-stained semi-thin section of control fat body tissue of young adult embedded in Epon is shown in F. Experiments presented in A–D were performed with fat body tissue of the same individual. Superficial cell borders (arrowheads), a nucleus (N), and several lipid droplets (L) are indicated.

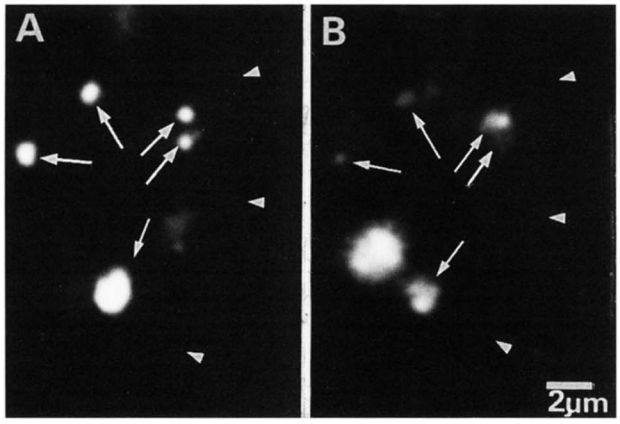

Fig. 2. Co-localization of DiI-HDLp and Cascade Blue hydrazide. Fat body tissue of young adult, 4 days after the imaginal ecdysis, was incubated for 90 min at 30°C simultaneously with 0.01 mg/ml DiI-HDLp and 10 mg/ml Cascade Blue hydrazide. Vibratome sections (20 μm) of agar-embedded tissue were examined with a fluorescence microscope for the localization of DiI-HDLp (A) and Cascade Blue (B), with appropriate filter settings for the different dyes. Co-localization of the two dyes (arrows) and the superficial cell border are indicated (arrowheads).

Cascade Blue hydrazide revealed that the two labels co-localized in intracellular spots (**Fig. 2**). This clearly indicates that the intracellular staining pattern observed after incubation with DiI-HDLp is due to lipoproteins that are localized within the endocytotic compartment of the fat body cells.

Together these experiments demonstrate that the lipoproteins are internalized by fat body cells of young adults by means of receptor-mediated endocytosis.

Endocytosis of HDLp in relation to developmental stage and after starvation

In the light of the previously postulated redistribution of HDLp binding sites during development (11), we compared receptor-mediated uptake of DiI-HDLp by fat bodies from the last two larval stages preceding imaginal ecdysis (fourth and fifth larval instars) as well as young and older adults (4 and 15 days after the imaginal ecdysis). In contrast to larvae (not shown) and young adults, where labeled spots were more or less equally distributed in the cytoplasm (**Fig. 3A**), fat body cells of older adults contained only a few labeled intracellular spots, which were predominantly located at the periphery of the cells (Fig. 3B). In addition, a much more intense fluorescent staining was observed at the surface of fat body cells of older adults compared to young adults and larvae, which indicates that only minor amounts of HDLp are internalized and that the majority of the HDLp is located at the cell surface.

As endocytosis of lipoproteins may be related to the process of lipid storage in the fat body cells, we studied whether starvation could prolong the intracellular localization of DiI-HDLp in fat body cells during development. Therefore, the endocytotic activity of fat body tissue of older adults that had been starved from day 8 to day 14 after the imaginal ecdysis and then fed on day 15, 1 day prior to killing, was also determined and compared to the endocytotic activity in control animals of the same age that had been fed normally. The number of fluorescently stained spots in the fat body of starved adults (**Fig. 4B**) was dramatically increased compared to control animals (Fig. 4A). A very typical staining pattern was observed; internalized HDLp was located mainly at the periphery of the cells while the centre was devoid of fluorescence.

For both control and starved older adults, it was demonstrated similarly as described above for young adults, that the internalization of HDLp by fat body cells is receptor-mediated, sensitive to ammonium chloride, and much less sensitive to chloroquine (not shown).

Uptake of lipid and protein moieties of lipoproteins

In order to determine whether the fat body cells obtain lipids from the lipoproteins either by a selective lipid transport process or by the endosomal/lysosomal pathway, the uptake of DAG and apolipoproteins from HDLp by fat body tissue of young adults, 4 days after the imaginal ecdysis, was monitored. The fat body appeared to selectively take up [^{3}H]DAG from the lipoproteins without substantial concomitant accumulation of the apolipoproteins (**Fig. 5**).

Under the experimental conditions used, approximately 50% of the [^{3}H]DAG present in lipoproteins was transported into the fat body. The density of the lipoproteins in the medium after incubation was determined; net transport of lipids into the fat body was expected to result in an increase in the density of the lipoproteins. However, no such increase in lipoprotein density could be detected; in contrast, a small decrease in lipoprotein density was found (typical experiment; control lipoprotein: 1.14 g/ml, lipoprotein after incubation: 1.12 g/ml) suggesting that no net transport of DAG to the fat body had taken place (this decrease in the density of HDLp is visible in the control curve in Fig. 7).

Although no net transport could be established, it was obvious that the lipids were exchanged between fat body cells and lipoproteins by a selective transport mechanism, as only the lipids were taken up whereas the apolipoproteins of HDLp did not accumulate in the cells.

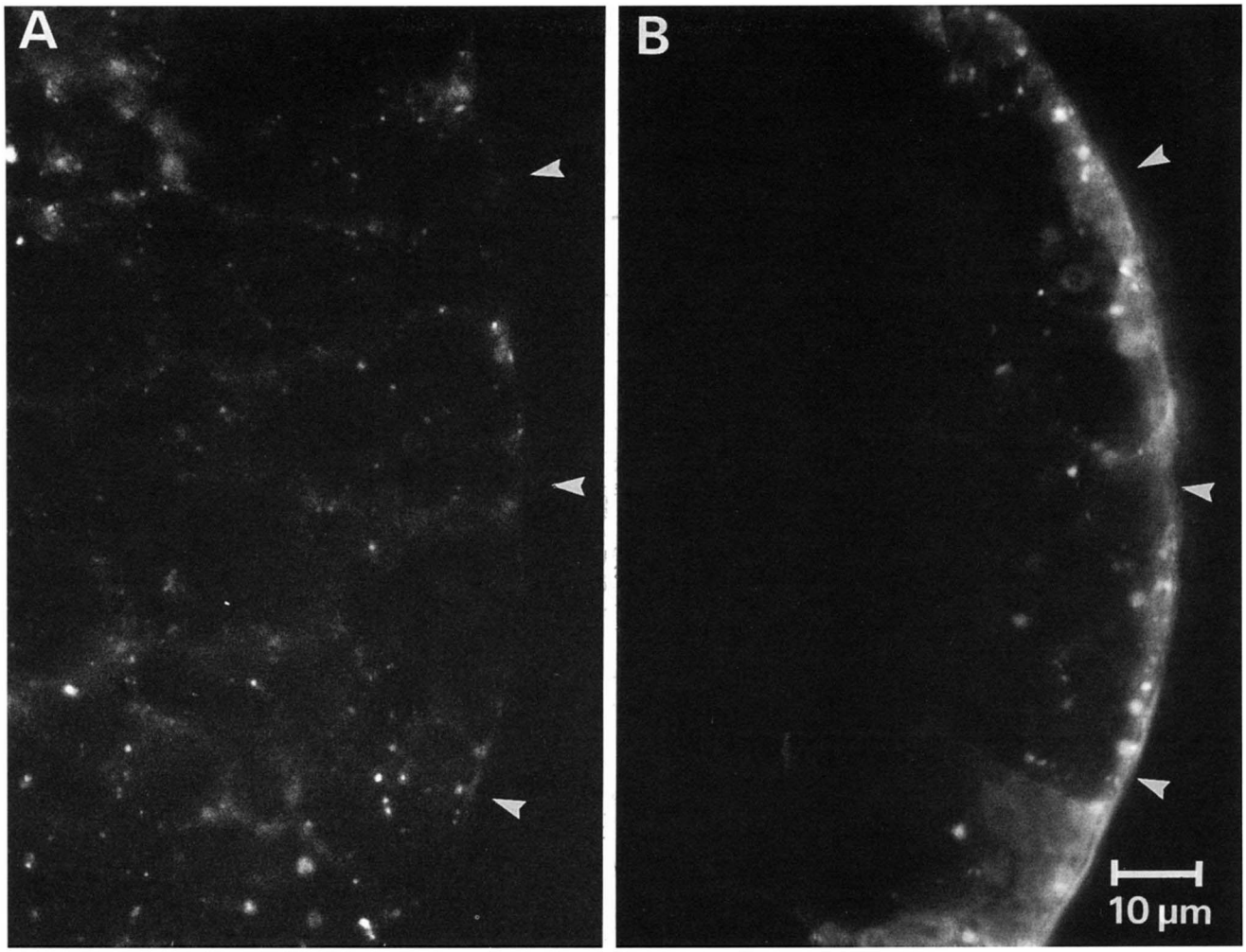

Fig. 3. Down-regulation of receptor-mediated endocytosis of HDLp during development. Fat body tissue of a young adult, 4 days after imaginal ecdysis (A), and of an older adult, 15 days after imaginal ecdysis (B), was incubated for 90 min at 30°C with 0.01 mg/ml DiI-HDLp. Vibratome sections (20 μm) of agar-embedded tissue were examined for the localization of labeled lipoproteins using fluorescence microscopy. The superficial cell borders (arrowheads) are indicated.

No substantial degradation of lipoproteins by fat body cells

The total amount of cell-associated lipoproteins and the amount of acid-soluble label, representing degraded lipoproteins, in fat body tissue and in the incubation medium were determined in young and older adults, 4 and 15 days after the imaginal ecdysis, after a prolonged incubation of 6 h with HDLp radioactively labeled in the protein moiety.

After this incubation, the amount of acid-soluble label in the medium, which accumulated throughout the incubation period, equaled 50% of the amount of total cell-associated lipoproteins when fat body tissue of young adults was used without additives (**Table 1**).

Only a very small fraction of the cell-associated label is attributed to degraded apolipoproteins: 3.9% of the labeled cell-associated lipoproteins was acid-soluble in the fat body tissue of young adults. Even significantly less cell-associated acid-soluble label was observed when fat body tissue of older adults was used. In addition, for young adults it was shown that the observed minimal degradation in the cell-associated label could be significantly inhibited by ammonium chloride. However, incubation of fat body tissue from young adults with lipoproteins in the presence of chloroquine, which is known to inhibit lysosomal degradation of internalized ligands (16), did not result in significantly fewer degradation products in the cell-associated label compared to the incubation without additives.

No significant differences in the amount of acid-soluble label in the medium were observed between the dif-

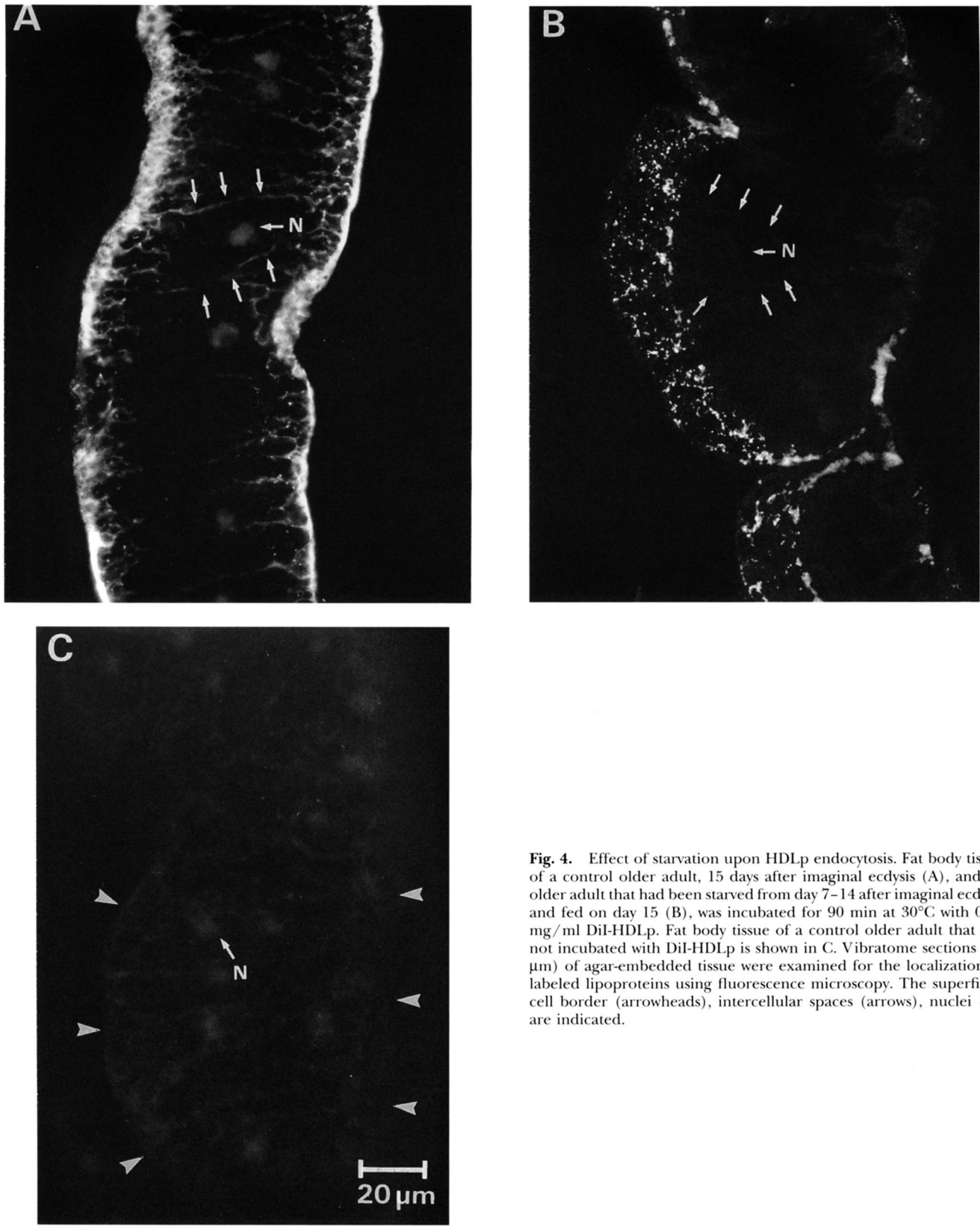

Fig. 4. Effect of starvation upon HDLp endocytosis. Fat body tissue of a control older adult, 15 days after imaginal ecdysis (A), and an older adult that had been starved from day 7–14 after imaginal ecdysis and fed on day 15 (B), was incubated for 90 min at 30°C with 0.01 mg/ml DiI-HDLp. Fat body tissue of a control older adult that was not incubated with DiI-HDLp is shown in C. Vibratome sections (20 µm) of agar-embedded tissue were examined for the localization of labeled lipoproteins using fluorescence microscopy. The superficial cell border (arrowheads), intercellular spaces (arrows), nuclei (N) are indicated.

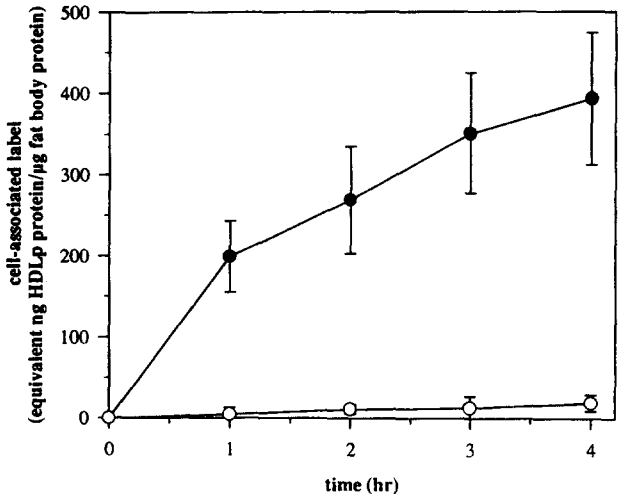

Fig. 5. Uptake of [^{3}H]DAG and [^{3}H]apolipoproteins from HDLp into the fat body. Fat body tissue of young adults, 4 days after the imaginal ecdysis, was incubated at 30°C with 0.1 mg/ml HDLp containing [^{3}H]DAG (closed circles) or [^{3}H]apolipoprotein (open circles). At the time points indicated, aliquots were taken from the incubation medium and the radioactivity in the aliquots was quantified using liquid scintillation counting. The decrease in the radioactivity content was regarded as cell-associated label. Data represent means ± SEM (n = 4).

ferent conditions and between the two developmental stages which may be due to the high variation in this parameter. Proteolytic activity originating from the dissected tissue and present in the incubation medium may be responsible for this variation and may in addition result in overestimation of the amount of degraded lipoproteins.

Endocytosis is not required for lipid exchange

Exchange of two lipid components of insect lipoproteins that are transported into the fat body, DAG (22) and cholesterol (23), was monitored in the presence and absence of ammonium chloride. Inhibition of endocytosis did not significantly affect DAG nor cholesterol exchange, implying that endocytosis is not a prerequisite for exchange of these two lipid components of HDLp (**Fig. 6**). The small decrease in lipoprotein density described above was shown to be independent of endocytotic activity because this shift in density occurred equally in the absence and presence of ammonium chloride (not shown).

Endocytosis is not required for LDLp formation

During sustained flight activity of insects, AKH-induced transport of large amounts of DAG from the fat body to the flight muscles occurs by conversion of HDLp at the fat body into the DAG-rich LDLp (4, 7). Using an in vitro incubation system, it was shown that endocytosis is not required for DAG mobilization after AKH stimulation, as ammonium chloride did not reduce the ability of fat body tissue to convert HDLp into LDLp in response to AKH (**Fig. 7**). In addition, using fluorescence microscopy, no changes were observed in the limited endocytotic uptake of DiI-HDLp by fat body tissue of older adults, 15 days after the imaginal ecdysis, during in vitro AKH stimulation (not shown). This demonstrates that the process of LDLp formation does not involve endocytotic processing of lipoproteins.

DISCUSSION

Receptor-mediated endocytosis of HDLp

Tsuchida and Wells (24) demonstrated that the protein moiety of insect lipoproteins did not accumulate

TABLE 1. Cell association and degradation of HDLp

	Total Cell-Associated	Acid-Soluble	
		Cell-Associated	Medium
	ng HDLp protein/µg fat body protein		
Young adult, control	15.3 ± 3.3	0.60 ± 0.17	7.7 ± 2.1
Young adult + NH_4CL	12.6 ± 5.2	0.16 ± 0.08[b]	8.1 ± 2.7
Young adult + chloroquine	12.3 ± 5.8	0.32 ± 0.17	2.3 ± 0.6
Older adult, control	9.8 ± 2.5[a]	0.08 ± 0.08[a]	3.8 ± 1.0

Fat body tissue of young adults, 4 days after imaginal ecdysis, and older adults, 15 days after imaginal ecdysis, was incubated for 6 h at 30°C with 0.2 mg/ml HDLp radiolabeled in the protein moiety in the absence or presence of 10 mM NH_4Cl and 100 µM chloroquine. Subsequently, the tissue was homogenized and the amounts of total cell-associated label, of acid-soluble cell-associated label (in 10% trichloroacetic acid) and of acid-soluble label in the medium were determined. Data represent means ± SEM (n = 5).

[a]Significantly different from young adult control incubation (unpaired *t*-test, $P < 0.05$).

[b]Significantly different from young adult control incubation (paired *t*-test, $P < 0.05$).

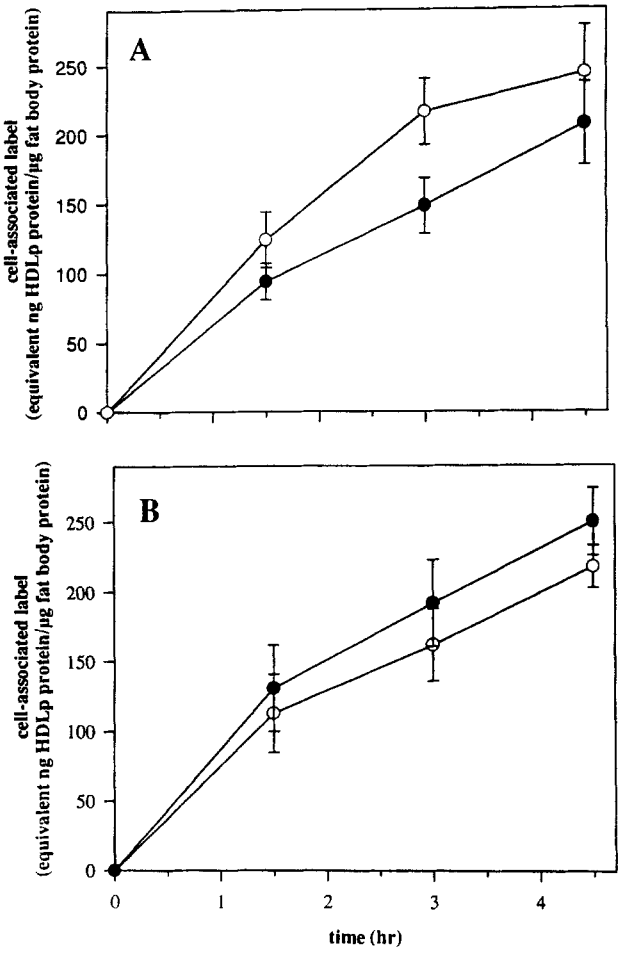

Fig. 6. Effect of inhibition of endocytosis on the uptake of [^{3}H]DAG and [^{3}H]cholesterol. Fat body tissue of young adults, 4 days after imaginal ecdysis, was incubated at 30°C with 0.2 mg/ml HDLp that had been radioactively labeled in the DAG (A) or cholesterol moiety (B). For both components their disappearance from the incubation medium was quantified by determining the radioactivity in aliquots of medium using liquid scintillation counting in the absence (closed circles) or presence (open circles) of 10 mM NH_4Cl. The radioactivity that had disappeared from the medium was regarded as cell-associated label. Data represent means ± SEM (n = 4).

in fat body tissue of a lepidopteran insect, which is in good agreement with the model of selective lipid transport mechanism facilitated by insect lipoproteins. Therefore, it seems unlikely that insect lipoproteins deliver their lipid moiety to the fat body by classical receptor-mediated endocytosis and subsequent trafficking to the lysosomes as described for other lipoproteins (25). Nevertheless, endocytosis of HDLp by the fat body of dragon fly larvae has been reported (26). In the present study, we demonstrate that the HDLp binding site at the locust fat body functions also as an endocytotic receptor and that HDLp is internalized by fat body cells by means of receptor-mediated endocytosis.

Although internalization of lipoproteins by fat body cells of young adults, 4 days after the imaginal ecdysis, was clearly shown, the vast majority of the lipoproteins apparently escaped from lysosomal hydrolysis. After a prolonged incubation (6 h) of fat body tissue of young adults with HDLp labeled in the protein moiety, the amount of degradation products of lipoproteins present in the incubation medium was approximately half of the amount of apolipoproteins bound to the fat body tissue. If efficient lysosomal degradation would occur, the incubation medium should contain several-fold the amount of cell-associated lipoproteins, as endocytotic receptors normally are able to carry out several cycles of delivery of ligands to the lysosomes during a period of 6 h. For example, for human low density lipoprotein it has been found that 5 times the amount of cell-associated lipoproteins is present in the medium in an acid-soluble form after 6 h (27). Also, the percentage of acid-soluble cell-associated radiolabeled lipoproteins, which is almost 4% for young adults, is much lower than the 16% observed for human low density lipoprotein. The low level of lysosomal degradation of lipoproteins did not result in a substantial accumulation of intact lipoproteins. Moreover, ammonium chloride, which strongly inhibits endocytosis of HDLp as discussed below, did not reduce the amount of cell-associated lipoproteins significantly, which confirms that no intracellular accumulation of lipoproteins takes place.

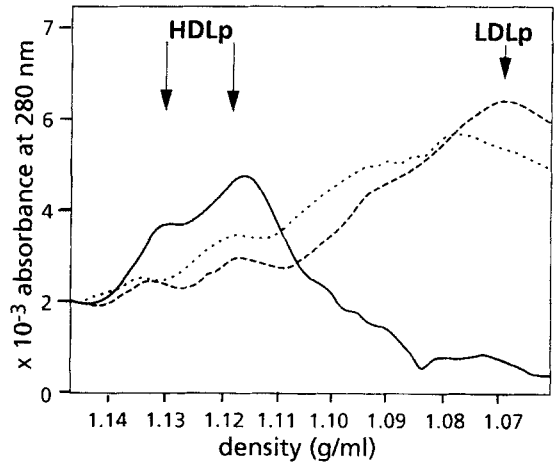

Fig. 7. Effect of inhibition of HDLp endocytosis on LDLp formation. Per experimental condition, 4 fat bodies of older adults, 15 days after imaginal ecdysis, were incubated with 1 mg HDLp at 30°C with 10 pmol AKH-I, as described previously (13), in the absence (dashed line) or presence (dotted line) of 10 mM NH_4Cl. Four non-stimulated fat bodies were taken as control (solid line). Subsequently, the media of the quadruplicate incubations per condition were pooled and subjected to density gradient ultracentrifugation. The gradients were fractionated during which the A_{280} was monitored. The arrows indicate the location of HDLp and LDLp. (The two HDLp populations indicated refer to the populations mentioned in Uptake of lipid and protein moieties of lipoproteins.)

In view of the absence of both substantial degradation and accumulation of HDLp in fat body cells, it is most likely that the lipoproteins are resecreted after internalization and intracellular processing. This pathway, known as retroendocytosis, has been described for several ligands of which transferrin is the classical example (28). A characteristic of ligands that are subject to retroendocytosis is that the ligand does not dissociate in response to the drop in pH within the lumen of the endosomes that occurs early during endosomal trafficking (15). In this context, it is noteworthy that the affinity of HDLp for its binding site increases when the pH decreases (10).

Ammonium chloride and chloroquine inhibit luminal acidification of endocytotic vesicles (15), which is a prerequisite for proper trafficking of several ligands. Consequently, these agents inhibit receptor-mediated endocytosis of many ligands (21), including the mammalian low density lipoprotein (29). In fat body cells, both ammonium chloride and chloroquine reduced the number of fluorescently stained spots. The inhibitory effect of ammonium chloride was accomplished at an early phase of endocytotic uptake as almost no intracellular staining was observed, suggesting that HDLp does not enter the cells. With chloroquine, however, only a diminished staining was observed at low HDLp concentrations while at higher concentrations the staining appeared to be similar to the pattern observed with control fat body tissue incubated without additives. Differences in the inhibitory activities of these two agents regarding intracellular sorting have also been reported by others (30), although generally they exhibit similar effects.

Down-regulation of endocytosis and coincidental increase of cell-surface association of HDLp

Endocytotic uptake of lipoproteins is strongly down-regulated during the adult stage; in older adults, 15 days after the imaginal ecdysis, the lipoproteins were located nearly exclusively at the superficial cell border of fat body cells. The few fluorescently stained spots present were, in comparison to young adults, located closer, to the superficial border of the cells. In a previous study, we observed that the total number of HDLp receptors of fat body cells appeared to be constant, whereas the number of HDLp receptors located at the plasma membrane increased during development (11). The observed down-regulation of endocytotic uptake of HDLp may provide an explanation for this observation as this process may result in a concomitant redistribution of receptors from the cell interior to the cell surface. Redistribution of intracellular receptors to the cell surface has been found for several membrane proteins expressed by mammalian cells. For example, the transferrin receptor (31), the glucose transporter (32), and type I insulin-like growth factor receptor (33) are redistributed from intracellular pools to the plasma membrane in adipocytes after hormonal stimulation.

The developmental decrease in endocytotic uptake of HDLp coincides with a reduction of lipid synthesis by fat body cells (34), implying a possible role for lipoprotein internalization in this process. The observation that fat body cells of starved adult locusts still showed substantial endocytotic uptake of HDLp at a developmental stage at which fat body cells of insects that were fed normally demonstrated almost no endocytosis, favors this possibility.

Down-regulation of internalization of HDLp appears to be accompanied by an increase in cell-surface association of these lipoproteins. Recent studies suggest that this increase in binding to the cell surface may be partly due to association of the lipoproteins to the extracellular matrix of fat body cells (N. P. Dantuma and M. A. P. Pijnenburg, unpublished results). Although binding studies with fat body tissue have shown that the cell surface binding is mainly attributable to binding of HDLp to a saturable high-affinity binding site, additional studies are required to distinguish between receptor-bound lipoproteins and lipoproteins encaptured by or attached to the extracellular matrix of adult fat body cells.

Function of receptor-mediated endocytosis of HDLp

Bauerfeind and Komnick (26) demonstrated endocytosis of HDLp by larval fat body cells and originally postulated that the function of this process could be either degradation of defective lipoproteins or lipid transport by a retroendocytotic pathway. In the light of the present study, the first hypothesis seems unlikely as we observed that endocytosis is strongly diminished during the adult stage, when the HDLp concentration in the hemolymph is considerably elevated (20). With respect to the second possibility, our biochemical experiments revealed that endocytosis of HDLp is not required for exchange of DAG or cholesterol between HDLp and fat body cells as this was not reduced by inhibition of endocytosis. In addition, endocytosis is not a prerequisite for the mobilization of lipids and their loading onto lipoproteins in response to AKH. Therefore, internalization of lipoproteins may mediate the transport of another component from or, more likely, to the fat body. DAG and cholesterol are lipids which theoretically can be exchanged by diffusion through the aqueous phase between the bound lipoprotein and the cell membrane (35). However, this is not an universal mechanism by which all lipids can be transferred as the efficiency of aqueous diffusion highly depends on the water solubility of the lipid. One reason for internalization of the

lipoproteins, therefore, may be that extraction of some minor lipid components, which cannot be transported by aqueous diffusion, can only be established after endocytotic uptake. The acidic environment in the endocytotic vesicles may be essential for this extraction process. Retroendocytosis has also been reported for mammalian high density lipoproteins (36–38) that, like insect lipoproteins, shuttle their lipid cargo between cells (1, 3, 39). Therefore, intracellular extraction during retroendocytotic trafficking of certain lipids from lipoproteins that deliver the majority of their cargo without internalization may be a general pathway in lipoprotein metabolism.

The occurrence of the ability to fly and, in addition, to mobilize lipids from the fat body cells in response to AKH (18) coincides with an increase in cell-surface association of HDLp (11). Recruitment of lipoproteins from the hemolymph to the surface of fat body cells is most likely required for loading of DAG onto the lipoproteins, which results in the formation of flight-specific LDLp. If down-regulation of endocytosis indeed results in a redistribution of HDLp receptors towards the plasma membrane, it might well be that during lipid mobilization these receptors may facilitate docking of the lipoproteins, thereby enabling efficient and rapid DAG transport.

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