Selective uptake of high density lipoprotein-associated cholesterylesters by differentiated Ob1771 adipocytes is modulated by endogenous and exogenous lipoprotein lipase

Frida Schorsch, Ernst Malle, Wolfgang Sattler*

Karl-Franzens University Graz, Department of Medical Biochemistry, Harrachgasse 21, A-8010 Graz, Austria

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Abstract The present study aimed at investigating mechanisms of selective uptake of HDL_3-associated cholesterylesters (HDL_3-CEs) by differentiated Ob1771 adipocytes. Our findings indicate that Ob1771 cells are capable of pronounced selective uptake of HDL_3-CEs in 2.6-fold excess of HDL_3 holoparticle uptake. Selective uptake of HDL_3-CEs into a releasable pool (presumably located in the cellular plasma membrane) was temperature insensitive while prominent internalization into a non-releasable and subsequent hydrolysis in a non-chloroquine sensitive compartment occurred at 37°C. Release of membrane bound endogenous LPL by heparin resulted in decreased HDL_3 holoparticle, total CE and selective CE uptake. Accordingly, the addition of exogenous LPL to the culture medium resulted in increased holoparticle, total CE and selective CE uptake.

Key words: Selective uptake; Adipocyte; HDL_3; Cholesterol; Lipoprotein; LPL

1. Introduction

Among peripheral tissues adipose tissue plays an important role for uptake, storage and mobilization of a large pool of cholesterol although the ability for endogenous cholesterol synthesis is limited [1]. For this reason adipose tissue is dependent on the interaction with interstitial lipoproteins to maintain cholesterol uptake [2]. In principle fat cells can acquire lipoprotein-associated cholesterol via specific plasma membrane binding sites for apoB, E and A containing lipoproteins [3–5]. HDL binding sites were identified in adipocytes of different origin and the affinity of these binding sites appears to be differentially regulated in cells obtained from obese and lean human subjects [6]. The binding capacity of these binding sites is regulated by the extracellular lipid composition present in the culture medium [7]. Besides the internalization of intact lipoprotein particles ('holoparticle uptake') via binding proteins HDL cholesterylesters (HDL-CEs) can be delivered to adipocytes by a mechanism termed selective uptake [8,9]. The term 'selective' refers to the fact that HDL-associated CEs can be taken up independently of holoparticle uptake, i.e. in excess of apolipoproteins [8,9].

Selective uptake of HDL-CEs was described for a variety of different cell lines and was also observed in vivo [10–12]. Selective uptake was thought to be an apolipoprotein independent process occurring via receptor independent pathways [13]. However, recently a scavenger receptor of the CD36 class (scavenger receptor BI, SR-BI) was identified as a HDL docking receptor mediating selective uptake of HDL-CEs [14]. In rats SR-BI is most abundantly expressed in adrenal gland and ovaries while the liver and epididymal fat contains only moderate amounts of SR-BI [14]. SR-BI expression in adipose tissue appears to be low [14] and vary to a certain degree [14,15], probably due to post-transcriptional regulation of SR-BI expression [15]. For these reasons one might anticipate that other factors than SR-BI mediate selective HDL-CE uptake in adipose tissue, a major cholesterol storage compartment in humans. A prominent candidate which could mediate such an effect is lipoprotein lipase (LPL) which is synthesized by adipocytes [16]. Besides its lipolytic activity [17] LPL has been shown to anchor lipoproteins to the surface of a variety of cells [18,19] thereby increasing surface binding and degradation of lipoproteins [20,21] by mechanisms involving the low density lipoprotein receptor related protein (LRP) and the very low density lipoprotein (VLDL) receptor [22–24]. In addition LPL was shown to enhance the efficacy of selective HDL-CE uptake by fibroblasts and macrophages [25,26].

To investigate whether selective uptake of HDL_3-associated CEs by differentiated Ob1771 adipocytes and subsequent hydrolysis could contribute to the maintenance of the intracellular cholesterol(ester) pool in adipocytes we have analyzed their ability for selective uptake of HDL_3-CEs, the temperature requirement for internalization, the effects of a lysosomal inhibitor on intracellular CE hydrolysis and the role of LPL during selective uptake of HDL-CEs.

2. Materials and methods

2.1. Cultivation of Ob1771 cells

Ob1771 preadipocytes were plated in 6 well trays at a density of 20 000 cells per well. Differentiation of confluent cells into mature adipocytes was performed in DMEM containing 10% FCS, insulin (17 nM) and triiodothyronine (2 nM) as described [27]. The medium was changed in three day intervals. The experiments reported below were performed between day 8–11 after differentiation where the cells have acquired small intracellular lipid droplets.

2.2. Preparation of human, apo E-free HDL_3

Human apo E-free HDL_3 was prepared by density gradient ultracentrifugation of plasma obtained from normolipemic donors in a TL120 Beckman tabletop ultracentrifuge as described [28]. SDS-PAGE on 15% gels and subsequent Coomassie staining revealed the presence of apo A-I as the major apolipoprotein.

*Corresponding author. Fax: +43 (316) 380-9615.
E-mail: wolfgang.sattler@kfunigraz.ac.at

Abbreviations: Apo, apolipoprotein; CE(s), cholesterylester(s); Ch, unesterified cholesterol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HDL_3, high density lipoprotein subclass 3; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline

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2.3. Lipoprotein labelling procedures

2.3.1. ^{125}I labelling of HDL_3. Iodination of HDL_3 was performed as described by Sinn et al. [29] using N-Br-succinimide as the coupling agent. Routinely 1 mCi of ^{125}INa (Amersham, Austria) was used to label 5 mg of HDL_3 protein. This procedure resulted in specific activities between 300–450 dpm/ng protein. Lipid-associated ('unspecific') activity was always less than 3% of total activity.

2.3.2. HDL_3 labelling with [^{3}H]Ch18:2. HDL_3 was labelled with [cholesteryl-1,2,6,7-^{3}H] linoleate (Du Pont NEN, Austria) or [cholesteryl-1,2-^{3}H] hexadecylether (a non-hydrolyzable CE analogue; Du-Pont NEN, Austria) by CETP-catalyzed transfer from donor liposomes as described previously [30]. This labelling procedure resulted in specific activities of 5–7 cpm/ng HDL_3 protein.

2.4. Uptake studies

Differentiated cells were incubated in the presence of increasing concentrations of radioactive ligands in the absence or presence of a 20-fold excess of unlabelled ligand to differentiate between total and non-specific binding. Specific binding values were calculated by subtraction of total minus non-specific binding. Uptake experiments were performed in DMEM containing LPDS (10%, v/v; 6 h, 37°C). Following this incubation the cells were washed twice in Tris-buffered saline (TBS) containing bovine serum albumin (5%, w/v) followed by three washes in TBS and measurement of the cell-associated radioactivity in NaOH (0.3 N) lysates. Where indicated chloroquine was added to the medium at a final concentration of 50 μM, 12 h prior to the uptake experiments [31]. To facilitate the comparison of results obtained with ^{125}I-HDL_3 and [^{3}H]Ch18:2-HDL_3 selective uptake of HDL_3-CE ([^{3}H]Ch18:2 minus ^{125}I) is expressed as apparent HDL_3 particle uptake (i.e. as the amount of apo A-I that would deliver the observed amount of tracer, if uptake were solely mediated by holoparticle uptake) as suggested by Pittman et al. [13]. The cellular protein content was measured according to Lowry [32] in NaOH lysates.

2.5. Lipid tracer analysis

To investigate whether adipocytes are able to hydrolyze HDL_3-associated CEs the cellular lipids were extracted after a incubation in the presence of [^{3}H]Ch18:2-HDL_3 with hexane/2-propanol (3:2, v/v) at room temperature on a plateau-shaker as described [33]. The lipid extracts were analyzed by HPLC and radiometric detection as described [26]. The mobile phase was methanol/2-propanol at a flow rate of 200 μl/min. The scintillation fluid flow rate was 1 ml/min. Peak identification of radioactive cellular lipids was performed by comparison with authentic external standards ([^{3}H]Ch and [^{3}H]Ch18:2).

2.6. Energy requirements for selective uptake

Differentiated adipocytes were incubated in the presence of 100 μg HDL_3 (labelled with [^{3}H]Ch-hexadecylether) at 4, 12, 22 and 37°C up to 4 h. At the indicated times cells were washed and chased in the presence of a 10-fold excess of unlabelled HDL_3 to dissociate the tracer from the 'releasable' compartment as described in [34]. The chase medium was removed and counted. The remaining cells were lysed in NaOH (0.3 N) to measure the cell-associated radioactivity ('non-releasable' pool) and the cellular protein content.

2.7. Effect of endogenous LPL on holoparticle and selective uptake

Uptake experiments were performed in DMEM/10% LPDS in the presence of ^{125}I-HDL_3 or [^{3}H]Ch18:2-HDL_3 (100 μg protein) either in the presence or absence of heparin (20 units/ml). Heparin releasable LPL activities were measured after a 30 min treatment of washed cells with 20 u/ml heparin as described in [26].

2.8. Effect of exogenous LPL on holoparticle and selective uptake

Bovine LPL was isolated as described [35] from fresh, unpasteurized bovine milk (1 l) and affinity chromatography on heparin- and phenyl-sepharose. The indicated concentrations were added together with the labelled HDL_3 preparations and incubated fin DMEM/10% LPDS. Thereafter the cells were washed as described above, lysed in NaOH and the cell-associated radioactivity was counted.

3. Results

3.1. HDL_3 holoparticle and HDL_3-CE uptake by differentiated Ob1771 cells

Holoparticle, total CE and selective uptake was measured with HDL_3 labelled either in the apoprotein (^{125}INa, holoparticle uptake) or in the lipid domain ([^{3}H]Ch18:2, total HDL_3-CE uptake). As we have not differentiated between bound and internalized radioactivity data shown in Fig. 1A–C represent total cell association of the respective HDL_3 preparation. HDL_3 holoparticle association (Fig. 1A) occurred at nearly linear rates over the HDL_3 concentration range used in these experiments. Incubations in the presence of [^{3}H]Ch18:2-labelled HDL_3 revealed that (at the highest HDL_3 concentrations used) total HDL_3-CE uptake exceeded HDL_3 holoparticle uptake 3.6-fold (Fig. 1B; 527 vs. 145 ng HDL_3 protein/mg cell protein). Consequently, HDL_3-CEs were taken up selectively in 2.6-fold excess of HDL_3 holoparticles (Fig. 1C). Non-linear regression analysis of specific total CE and selective CE association (Fig. 1C) revealed B_{max} values of 682 ± 44 and 453 ± 34 ng HDL_3 protein/mg cell protein and K_d values of 64 ± 10 and 42 ± 9 μg HDL_3 protein/ml/mg cell protein (total and selective CE uptake, respectively).

3.2. Temperature dependency of selective HDL_3-CE uptake into the releasable non-releasable cellular pool

Uptake efficacy of the [^{3}H]Ch-hexadecylether tracer into the releasable pool of adipocytes was very similar at 4, 12 and 22°C (Fig. 2A; 496, 467, and 457 ng HDL_3 protein/mg cell protein after a 6 h incubation, respectively). In contrast, incubations at 37°C resulted in a pronounced decrease of HDL_3-CEs located in the releasable pool (228 ng HDL_3 protein/ng cell protein at 6 h). Accordingly, the amount of tracer associated with the non-releasable, internalized pool showed the opposite tendency: At 4, 12 and 22°C the amount of tracer associated with the non-releasable pool was rather low (Fig. 2B; 78, 88 and 113 ng HDL_3 protein/mg cell protein after a 6 h incubation). At 37°C Ob1771 cells were capable of CE internalization and accumulated 508 ng HDL_3 protein/mg cell protein. These data demonstrate that selective uptake into the 'releasable' pool of adipocytes is temperature independent, while internalization into the 'non-releasable' pool is energy dependent, as expected for an internalization step.

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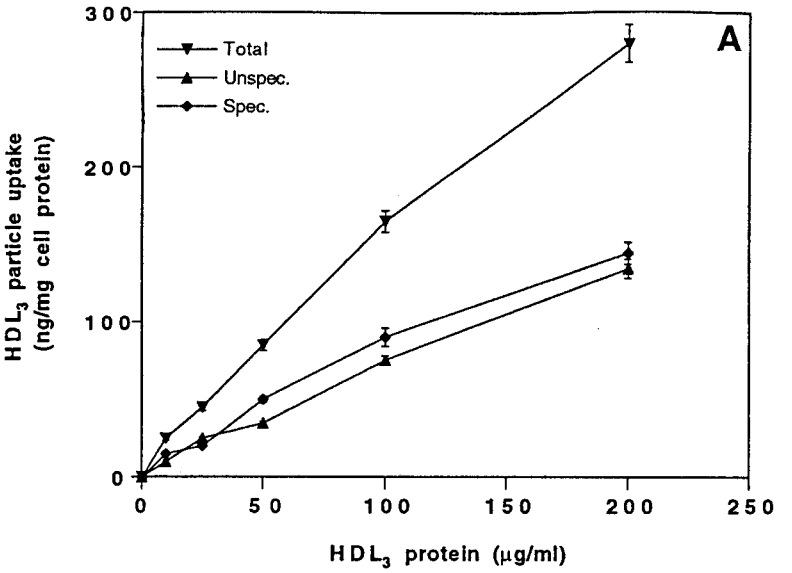
Fig. 1. HDL_3 holoparticle (A), total HDL_3-CE (B) and selective HDL_3-CE (C) uptake by differentiated Ob1771 adipocytes. Ob1771 cells were plated and differentiated into mature adipocytes as described in Section 2. 12 h prior to the uptake experiments the medium was switched to DMEM containing 10% LPDS. Cells were then incubated in the presence of the indicated concentrations of ^{125}I- and [^{3}H]Ch18:2-HDL_3. After 6 h the cells were washed and lysed in NaOH to measure the cell-associated radioactivity and the cellular protein content. ^{125}I uptake represents HDL_3 holoparticle uptake (A), while uptake of [^{3}H]Ch18:2 represents total HDL_3-CE uptake (B). Selective uptake (C) was calculated as the difference between specific [^{3}H]Ch18:2 and ^{125}I-HDL_3 uptake. Uptake is shown as apparent HDL_3 particle uptake (expressed as HDL_3 protein that would be necessary to account for the observed tracer uptake; see Section 2). Values shown represent mean ± S.D. from triplicate dishes from one representative experiment.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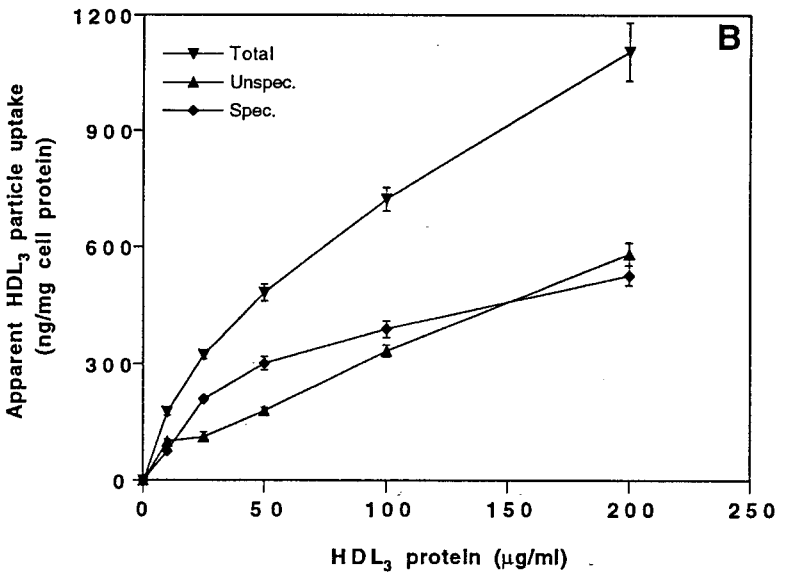
B
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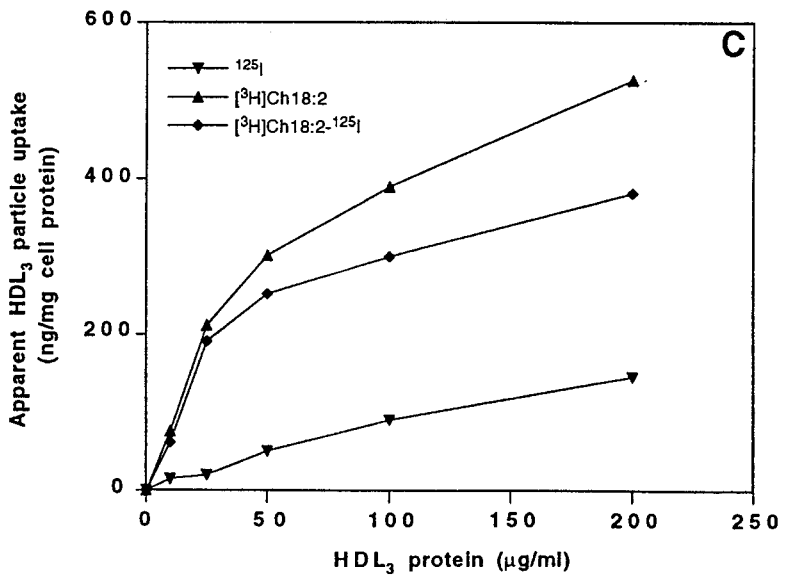
C
125I
[3H]Ch18:2
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Apparent HDL3 particle uptake (ng/mg cell protein)
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HDL3 protein (μg/ml)

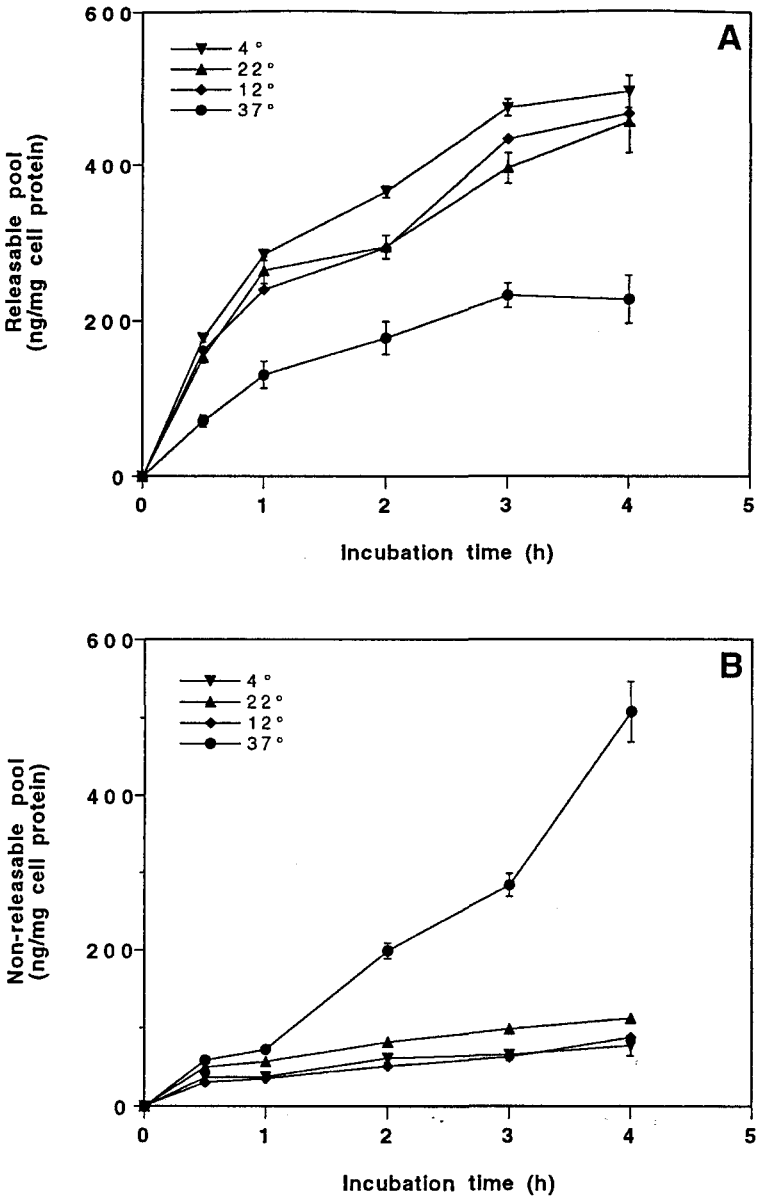

Fig. 2. Temperature dependency of selective HDL_3-CE uptake by Ob1771 adipocytes. Cells were plated on 35 mm petri dishes, differentiated as described in Section 2 and incubated in the presence of 200 μg HDL_3 (labelled with [^{3}H]Ch-hexadecylether, a non-hydrolyzable CE analog) at 4, 12, 22 and 37°C. At the indicated time points the cells were washed with BSA containing TBS, followed by two washes with albumin-free TBS. The tracer located in the plasma membrane (releasable pool, A) was chased (2 h) into the medium by the addition of a 10-fold excess of unlabelled HDL_3. Subsequently the chase medium was removed and counted. The cells were then washed as described above, lysed in NaOH and the remaining cell-associated activity was counted (referred to as non-releasable pool, B). Data shown represent mean ± S.D. from triplicate dishes from a typical experiment.

3.3. *Effect of chloroquine on intracellular hydrolysis of HDL_3-CEs*

In the next set of experiments we intended to assess whether CEs taken up by Ob1771 cells via the selective uptake pathway are subjected to intracellular hydrolysis. To address the question whether CE hydrolysis occurs in a lysosomal or non-lysosomal compartment, uptake experiments were performed in the absence and presence of the lysosomal inhibitor chloroquine [31]. Experiments performed in the absence (Fig. 3A) or in the presence (Fig. 3B) of chloroquine gave nearly identical results for intracellular hydrolysis rates at all time points analyzed (e.g. after an 8 h incubation we have observed Ch18:2:Ch ratios of 1.48 (control) and 1.58 (50 μM chloroquine). Also on a quantitative basis the radioactivity accumulated by the cells was nearly identical with 1418 ± 174 vs. 1387 ± 74 cpm present in the CE fraction and 957 ± 133 vs. 868 ± 93 cpm in the cholesterol fraction (Fig. 3A vs. Fig. 3B, 8 h incubation). Hydrolysis yield of intracellular CEs increased in a time dependent manner from 26 (1 h) to 40% (8 h).

3.4. *Effect of endogenous and exogenous LPL on the capacity for selective HDL_3-CE uptake*

To clarify the role of endogenous LPL during selective HDL-CE delivery uptake studies with ^{125}I-HDL_3 and [^{3}H]Ch18:2-HDL_3 were performed in the absence or presence

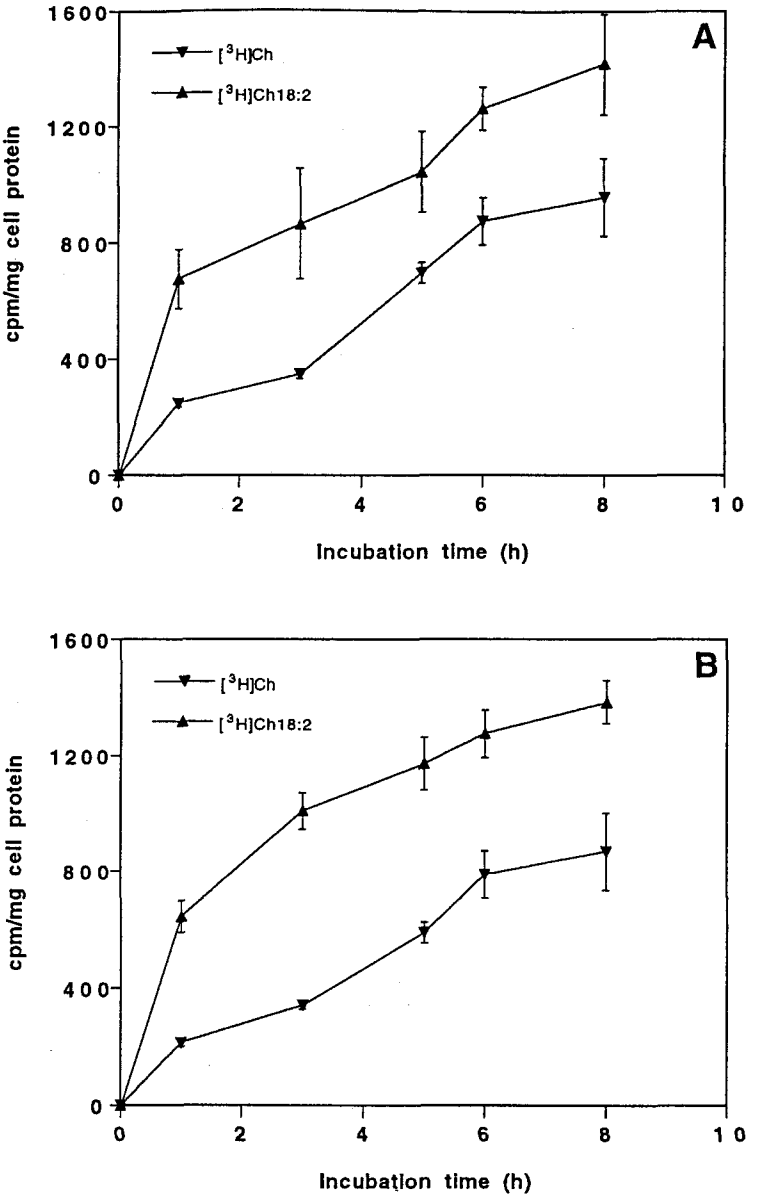

Fig. 3. Effect of chloroquine on the intracellular hydrolysis of HDL_3-associated [^{3}H]Ch18:2. Cells were plated on 6 well trays and differentiated as described in Section 2. Cells were pre-cultured in the absence (A) or presence (B) of chloroquine (50 μM; 12 h) and received then fresh medium containing [^{3}H]Ch18:2-labelled HDL_3 (50 μg) and chloroquine (50 μM). After a 8 h incubation the cellular lipids were extracted into hexane/2-propanol (3:2, v/v), dried and redissolved in methanol/2-propanol (200 μl). 20 μl of the extracts were analyzed by reversed phase HPLC with radiometric detection to measure the radioactivity present as [^{3}H]Ch and [^{3}H]Ch18:2. Data shown represent mean ± S.D. values from one experiment performed in triplicates.

of heparin to release membrane bound LPL. The heparin releasable LPL activity in Ob1771 cells was 24.2 nmol FFA/min/mg cell protein. The addition of heparin reduced HDL_3 holoparticle uptake by 15% (Table 1) as compared to experiments performed in the absence of heparin (111 ± 9.1 vs. 94 ± 3.9 ng HDL_3 protein/mg cell protein). The effect of heparin treatment of Ob1771 cells was somewhat more pronounced for total HDL-CE uptake and resulted in a reduction by 21%, while selective uptake of HDL_3-CEs was reduced by 23% in the presence of heparin (Table 1).

The effects of exogenous LPL on holoparticle, total CE and selective CE uptake is shown in Table 2. In this set of experiments cells were coincubated with ^{125}I-HDL_3 or [^{3}H]Ch18:2-labelled HDL_3 (100 μg) in the absence or presence of 50 μg LPL (molar ratio LPL:HDL_3 = 0.5). The addition of exogenous LPL resulted in a pronounced increase of cell-associated radioactivity. Upon addition of 50 μg of bovine LPL to the culture medium along with the correspondingly labelled HDL_3 preparation specific holoparticle association was increased 1.3-fold, total CE uptake increased 1.5-fold and selective uptake was increased 1.6-fold.

4. Discussion

During the present study we intended to clarify some aspects of selective HDL_3-CE uptake by differentiated Ob1771 adipocytes. The permanent Ob1771 cell line was chosen to exclude variations in HDL uptake/binding capacity as ob-

served for human fat cells which are partially related to differences in cell size [36]. In vivo, adipose tissue represents a major cholesterol storage organ, with the majority present in the unesterified ('free') form. In principle cholesterol can be delivered to fat cells either associated with different lipoprotein classes. However, Despres et al. [8] have demonstrated that the cellular processing of LDL and HDL by adipocytes differs remarkably: while human adipocytes are able to bind HDL subclasses in a specific and saturable manner degradation of HDL is low. In contrast, LDL is degraded very efficiently by these cells [8]. This could indicate that the interaction of HDL with adipocytes serves (a) different physiological function(s) from LDL and among these selective delivery of HDL-CEs to fat cells might represent a quantitatively important pathway for cholesterol(ester) delivery. Findings of elevated post-heparin LPL activities and reduced HDL cholesterol levels in obese subjects could indicate that LPL (in addition to other uptake mechanisms) is involved in HDL lipid uptake by adipose tissue [37].

During the present study we have observed pronounced selective uptake of HDL-CEs by Ob1771 cells. This effect was correlated with HDL_3 holoparticle binding/uptake, an indication that the two processes are not completely independent of each other, in line with the presence of HDL-binding proteins on adipocytes [38–40]. Qualitatively similar findings have been reported for human fat cells, however, on a quantitative basis the capacity for selective HDL-CE uptake by human adipocytes appears to be much higher, exceeding HDL_3 holoparticle uptake almost 20-fold [8]. The first step of selective uptake is the transfer of HDL-CEs into a plasma membrane-associated cellular pool (termed 'releasable', i.e. chase-accessible pool; [34]) occurring prior to irreversible internalization and subsequent hydrolysis [31]. To investigate whether uptake of HDL-CEs into the releasable pool is energy dependent (e.g. dependent on enzymatically active LPL) chase experiments were performed at temperatures between 4 and 37°C. In line with findings by Reaven et al. [41] we have observed that uptake in the releasable, plasma membrane-associated pool occurred even at 4°C. However, in contrast to our findings Reaven et al. [41] have reported that internalization of fluorescent CE analogs by granulosa cells occurred only upon warming of the cells. Results from the present study indicate that a portion of [^{3}H]Ch-hexadecylether was not dissociable from the cells. This could indicate that some CEs are transferred in the non-releasable pool also at low temperatures. Alternatively, altered membrane fluidity of the Ob1771 cells at low temperatures could explain inadequate chase yields. However, the majority of internalization occurred at 37°C, as expected for an internalization step.

Table 1
Effect of heparin on HDL_3 holoparticle association (^{125}I-HDL_3), total HDL_3-CE ([^{3}H]Ch18:2-HDL_3) and selective HDL_3-CE (^{3}H-^{125}I) uptake by Ob1771 adipocytes

	ng HDL_3 protein/mg cell protein	
	Control	Heparin (20 u/ml)
^{125}I-HDL_3	111 ± 9.1	94 ± 3.9
[^{3}H]Ch18:2-HDL_3	472 ± 27.0	373 ± 19.5
^{3}H-^{125}I	361 ± 17.2	279 ± 13.9

Cells were plated and cultured in 6 well trays as described in Section 2. Heparin (20 u/ml) was added simultaneously with 100 μg of [^{3}H]Ch18:2-labelled HDL_3 in the absence and presence of unlabelled HDL_3 and left on the cells for 6 h. Cells were washed, lysed and the cell-associated radioactivity and the cellular protein content was estimated. Data shown represent specific, cell-associated activity (mean ± S.D. from triplicate dishes from one representative experiment).

Table 2
Effect of exogenous lipoprotein lipase on HDL_3 holoparticle association (^{125}I-HDL_3), total HDL_3-CE ([^{3}H]Ch18:2-HDL_3) and selective HDL_3-CE (^{3}H-^{125}I) uptake by Ob1771 adipocytes

	ng HDL_3 protein/mg cell protein	
	Control	LPL (50 μg/ml)
^{125}I-HDL_3	101 ± 12.0	131 ± 13.4
[^{3}H]Ch18:2-HDL_3	452 ± 27.9	678 ± 42.5
^{3}H-^{125}I	351 ± 17.2	547 ± 33.1

Differentiated cells were washed and received fresh medium containing 100 μg [^{3}H]Ch18:2-labelled HDL_3 together with 50 μg of purified bovine LPL. After a 6 h incubation the cells were washed and the specific, cell-associated radioactivity was counted. Data shown represent mean ± S.D. of specific cell-associated activities from triplicate dishes from one representative experiment.

Following internalization into the non-releasable pool HDL-CEs taken up via the selective uptake pathway are subjected to intracellular hydrolysis. This process was demonstrated in a variety of different cell types [26,30,31,42–44]. Results from the current study demonstrate that Ob1771 cells are able to hydrolyze CEs which were taken up selectively. In line with other findings [30,31,44] CE hydrolysis was insensitive to chloroquine, indicating hydrolysis in an extralysosomal compartment. Interestingly the efficacy of CE hydrolysis in Ob1771 cells appeared to be much lower as compared to e.g. mouse peritoneal macrophages: while in the latter cells HDL-CEs are hydrolyzed almost quantitatively within minutes after the addition of [^{3}H]Ch18:2-HDL [26], only 40% of [^{3}H]Ch18:2 which was taken up selectively were hydrolyzed within 8 h by Ob1771 cells. This could reflect lower activities of the nCEH responsible for extralysosomal hydrolysis [45] and/or transfer into a compartment (probably the lipid droplet) which renders CEs less accessible to the action of hydrolytic enzymes.

Earlier reports [25,26] indicated that exogenous LPL significantly increases (selective) HDL-CE uptake, most probably a result of the bridging function of the enzyme. In contrast, endogenous LPL appeared to be without effect on selective HDL-CE delivery to different cell types [26,42,43]. Results obtained during the present study demonstrated that endogenous, membrane bound (i.e. heparin releasable) LPL is in part responsible for selective HDL_3-CE uptake. This is most probably a reflection of higher endogenous LPL synthesis in Ob1771 cells. While heparin treatment of Ob1771 adipocytes released 24 nmol FFA/min/mg cell protein the corresponding value in mouse peritoneal macrophages was almost 6-fold lower (on average 4 nmol FFA/min/mg cell protein; [26]). Independent whether the effects of endogenous or exogenous LPL on selective uptake were investigated the mechanism(s) of LPL-enhanced selective uptake appears to be similar in different cell types: LPL induces a significant increase in HDL holoparticle association/binding to the plasma membrane, thereby enhancing the probability for selective HDL-CE uptake. In line with a more general role of lipases in cholesterol homeostasis it is important to note that endogenous as well as exogenous bile salt stimulated cholesterol esterase was demonstrated to enhance selective uptake of HDL-CEs by HepG2 cells [46].

Hydrolysis of HDL-CEs taken up via the selective uptake pathway by a nCEH [45] could generate substantial amounts of intracellular FFAs. It was demonstrated that FFAs (in addition to other agents/factors) regulate LPL gene expression and enzyme activity [47,48]. This regulatory effect of FFAs occurs most probably via PPAR activation [48]. It was proposed that the regulation of LPL by FFA might represent a fine tuning step for the entry of fatty acids into adipocytes during fasting/feeding periods [48]. It is tempting to speculate that FFAs generated by extralysosomal hydrolysis of originally HDL-associated CEs which were subjected to selective uptake by adipocytes (in addition to FFAs liberated from TGs) could contribute to the regulation/modulation of LPL expression and/or activity thus providing an important in vivo function.

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