High-density lipoprotein (HDL$_3$)-associated α-tocopherol is taken up by HepG2 cells via the selective uptake pathway and resecreted with endogenously synthesized apo-lipoprotein B-rich lipoprotein particles

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α-Tocopherol (αTocH) is transported in association with lipoproteins in the aqueous milieu of the plasma. Although up to 50 % of circulating αTocH is transported by high-density lipoproteins (HDLs), little is known about the mechanisms of uptake of HDL-associated αTocH. During the current study, human apolipoprotein (apo)E-free HDL subclass 3 (HDL$_3$) labelled with [^{14}C]αTocH was used to investigate uptake mechanisms of HDL$_3$-associated αTocH by a permanent hepatoblastoma cell line (HepG2). HDL$_3$-associated αTocH was taken up independently of HDL$_3$ holoparticles in excess of apoA-I comparable with the non-endocytotic delivery of cholesteryl esters to cells termed the 'selective' cholesteryl ester uptake pathway. Experiments with unlabelled HDL$_3$ demonstrated net mass transfer of αTocH to HepG2 cells. Time-dependent studies with [^{14}C]αTocH-labelled HDL$_3$ revealed tracer uptake in 80-fold excess of apoA-I and in 4-fold excess of cholesteryl linoleate. In addition to HLDs, low-density lipoprotein (LDL)-associated αTocH was also taken up in excess of holoparticles, although to a lesser extent. These findings were confirmed with unlabelled lipoprotein preparations, in which HDL$_3$ displayed a 2- to 3-fold higher αTocH donor efficiency than LDLs (lipoproteins adjusted for equal amounts of αTocH). An important factor affecting particle-independent uptake of αTocH was the cellular cholesterol content (a 2-fold increase in cellular cholesterol levels resulted in a 2.3-fold decrease in uptake). Pulse–chase studies demonstrated that some of the HDL$_3$-associated αTocH taken up independently of holoparticle uptake was resecreted along with a newly synthesized apoB-containing lipoprotein fraction.

INTRODUCTION

Vitamin E is a generic term for the naturally occurring tocopherols and tocotrienols, and, of these, α-tocopherol (αTocH) has the highest biological activity [1]. *In vivo*, vitamin E is an important constituent of cellular membranes in all tissues, acting as a major lipid-soluble chain-breaking antioxidant and thereby protecting unsaturated fatty acid residues against (per)oxidative damage [2]. It is an essential nutrient, and the major symptoms of vitamin E deficiency are neurological dysfunction, muscular weakness and reproductive failure [3,4].

Dietary vitamin E is taken up by intestinal cells and resecreted in chylomicrons in proportion to the concentration of the naturally occurring tocopherols in the diet [5]. *In vivo* studies have demonstrated preferential enrichment of very-low-density lipoproteins with αTocH over γTocH, a process facilitated by an hepatic α-TocH-binding/transfer protein (reviewed in ref. [6]). In the fasting state, αTocH is distributed between the very-low-, low- and high-density lipoprotein (VLDL, LDL and HDL respectively) fraction in a 1:1.9:1.4 proportion [7]. However, this distribution is dependent on the relative lipoprotein content of the plasma samples analysed [7]. In the fed state, αTocH uptake by peripheral tissues may occur by different pathways. One of the first steps contributing to tissue supply with αTocH is lipoprotein lipase-mediated hydrolysis of triacylglycerol-rich lipoproteins [8]. Both the lipolytic activity and the bridging function of lipoprotein lipase appear to contribute to lipid transfer from lipoproteins to cells [9] or tissues [10]. Secondly, tissues may partially acquire LDL-associated αTocH via the LDL-receptor pathway. However, the quantitative importance of this pathway is not entirely clear, since Watanabe heritable hyperlipidaemic rabbits (lacking functional LDL receptors) have normal tissue αTocH concentrations [11]. Finally αTocH transfer between lipoproteins and tissues may contribute to the maintenance of tissue αTocH levels.

As a result of the oxidative theory of atherogenesis [12], LDL-associated αTocH has received much attention, especially with regard to its anti- and/or pro-oxidative properties during radical-initiated lipid peroxidation of LDL [13,14]. HDL-associated αTocH has received much less attention, a surprising fact since 30–50 % of circulating αTocH is carried within the HDL fraction [7,10]. Consequently, the mechanisms of HDL-associated αTocH uptake are poorly understood and have been attributed to non-specific receptor-independent transfer mechanisms [15]. In contrast with LDL-associated lipids, which are primarily metabolized in association with intact lipoprotein particles via the LDL-receptor pathway, HDL lipids [particularly cholesteryl esters (CEs)] are turned over by three different metabolic pathways: (i) metabolism of intact HDL particles (holoparticle uptake [16]); (ii) CE-transfer protein (CETP)-mediated exchange [17]; (iii) a pathway termed selective uptake [18]. 'Selective' refers to the fact that CEs are extracted from the HDL particle and internalized via scavenger receptor BI (SR-BI)-mediated mechanisms without concomitant endocytosis of the remaining HDL particle [19].

As 'selective uptake' has turned out to be an important metabolic pathway for HDL-associated lipids, we investigated whether the uptake of HDL-associated αTocH by HepG2 cells is

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mediated in the same way as selective uptake of HDL-associated CEs; both the polarity and topographical location [20] of αTocH within the HDL particle are compatible with such a concept. Subsequently we addressed the question of whether there are regulatory steps in common with the selective uptake of HDL-associated CEs, and, if uptake does occur via the selective uptake pathway, is αTocH resecreted along with endogenously synthesized lipoproteins? We finally looked at whether there are differences between HDL$_3$ and LDL with regard to their αTocH-donor efficacy.

EXPERIMENTAL

Materials

Chloroquine, monensin, NaN$_3$, KNO$_3$ and egg yolk lecithin were from Sigma, Vienna, Austria. Methanol, propan-2-ol, hexane, KBr, Na$_2$MoO$_4$ and (NH$_4$)$_2$VO$_3$ were obtained from Merck, Vienna Austria. LC-18 reversed-phase HPLC columns were obtained from Supelco, Vienna, Austria (250 mm $\times$ 4.5 mm) or Chrompack, Vienna Austria (200 mm $\times$ 2 mm). [^{14}C]αTocH was a gift from Dr. W. Steiling, Henkel Co., Dusseldorf, Germany. All other radiochemicals were obtained from NEN, Vienna, Austria. Consumables used during cell culture studies were obtained from BioWhitaker or Costar Vienna, Austria. PD-10 size-exclusion columns were from Pharmacia, Uppsala, Sweden, and immunoprecipitin was obtained from Gibco, Paisley, Scotland, U.K. All other materials were purchased from Merck, Darmstadt, Germany as analytical-grade reagents.

Lipoprotein preparation

Human LDL and apolipoprotein (apo)E-free HDL$_3$ were prepared by density-gradient ultracentrifugation of plasma obtained from normolipidaemic donors in a TL120 tabletop ultracentrifuge (at $r_{av} \approx 350\,000\ g$; Beckman, Vienna, Austria) [21] or by preparative discontinuous ultracentrifugation [22]. HDL$_3$ was recovered from the tubes, and excess KBr was removed by size-exclusion chromatography on PD-10 columns. SDS/PAGE revealed the presence of apoA-I as the major apolipoprotein. Lipoprotein-deficient serum (LPDS) was prepared by standard techniques [23].

Lipoprotein labelling

HDL$_3$ labelling with [*cholesteryl*-1,2,6,7-^{3}H]linoleate ([^{3}H]-Ch18:2), was achieved by CETP-catalysed transfer from donor liposomes [24]. Briefly, 200 μCi of [^{3}H]Ch18:2 and 80 μg of egg yolk lecithin were dried under argon and sonicated in 1 ml of PBS, pH 7.4. HDL$_3$ (1 ml, containing 2–5 mg of protein), LPDS (1 ml, as a source of CETP) and PBS (1 ml) were added. The mixture was incubated overnight under argon at 37 °C in a shaking water-bath. For the sake of comparability, labelling of HDL$_3$ with [1,2,6,7-^{3}H]cholesterol ([^{3}H]Ch) or D-α-5-methyl[^{14}C]-tocopherol ([^{14}C]αTocH) was performed exactly as described for [^{3}H]Ch18:2. Subsequently the labelled HDL$_3$ was reisolated in a TLX120 bench-top ultracentrifuge in a TLA100 rotor (Beckman) as described [24]. The HDL$_3$ band was aspirated and desalted by size-exclusion chromatography on PD-10 columns. This labelling procedure resulted in specific radioactivities of 0.5–3, 5–7 and 8–10 c.p.m./ng of HDL$_3$ protein for [^{14}C]αTocH, [^{3}H]Ch18:2 and [^{3}H]Ch respectively.

To compare transfer of [^{14}C]αTocH and net mass transfer of endogenous lipoprotein associated αTocH to HepG2 cells, HDL$_3$ (1.5 mg of protein, final volume 2 ml in PBS) or LDL (0.6 mg of protein, final volume 2 ml in PBS) was labelled by direct injection

of increasing specific radioactivities of an ethanolic [^{14}C]αTocH solution and incubation at 37 °C (3 h; under argon; shaking water-bath). Non-lipoprotein-associated [^{14}C]αTocH was removed by size-exclusion chromatography on PD-10 columns. Lipids were extracted and analysed by radio-HPLC as described below. To allow simultaneous quantification of tracer and endogenous αTocH, the radiometric detector was preceded by a fluorescence detector. To compare transfer of tracer and endogenous αTocH, HDL$_3$ and LDL preparations with similar specific radioactivities were chosen from the set of labelled lipoproteins prepared as described above.

HDL$_3$ and LDL were iodinated as described by Sinn et al. [25] using N-Br-succinimide as the oxidizing agent. Routinely 1 mCi of Na^{125}I was used to label either 5 mg of HDL$_3$ protein or 2 mg of LDL protein. This procedure resulted in specific radioactivities of between 300 and 450 d.p.m./ng of protein. Lipid-associated radioactivity was always less than 3 % of total radioactivity. No cross-linking or fragmentation of apoA-I or apoB-100 due to the iodination procedure was detected by SDS/PAGE and subsequent autoradiography.

αTocH analysis

Cellular lipids were extracted with hexane/propan-2-ol (3:2, v/v) at 25 °C (30 min on a plateau shaker) [26]. The lipid extracts were dried under argon, redissolved in 200 μl of methanol and analysed by reversed-phase HPLC on an LC-18 column (25 cm $\times$ 0.46 cm) with methanol as the mobile phase (1 ml/min) and fluorescence detection (excitation and emission wavelengths 292 and 335 nm respectively). αTocH was quantified by peak-area comparison with external standards of known concentration. The concentration of freshly prepared standards was estimated photometrically ($\epsilon_{292} = 3467\ \mathrm{M^{-1} \cdot cm^{-1}}$).

Reversed-phase HPLC of radiolabelled αTocH and metabolites was performed on a Chrompack LC-18 column (250 mm $\times$ 2 mm) with methanol/water (98:2, v/v) as the mobile phase (200 μl/min), essentially as described [27]. Radiolabelled compounds were detected with a Radiomatic Flo-One radiometric HPLC detector (Packard-Canberra; channel set at 0–156 keV) which was preceded by a fluorescence detector to allow simultaneous detection of labelled and unlabelled compounds. Liquid-scintillation fluid flow rate was set to 1 ml/min.

Cell culture studies

Human hepatoma HepG2 cells [28] were seeded in 35 mm multiwell culture plates or on 35 mm Petri dishes and cultured under standard conditions (37 °C; 5 % CO$_2$; 95 % humidity) in Dulbecco's modified Eagle's medium (DMEM) containing fetal calf serum (FCS; 10 %, v/v), streptomycin (40 μg/ml) and penicillin (40 units/ml). When the cells were 70–80 % confluent, the monolayers were washed twice with Tris-buffered saline (TBS, pH 7.4) and conditioned in fresh DMEM containing either FCS (10 %, v/v) or LPDS (10 %, v/v) for 24 h. The cells were then washed again (DMEM, 2 $\times$) and the uptake assays were initiated in DMEM containing either FCS or LPDS.

For uptake studies, HepG2 cells were incubated for the indicated times with the corresponding radiolabelled HDL$_3$ preparations in the absence or presence of excess (20-fold) unlabelled HDL$_3$, and, where noted, the appropriate amount of metabolic inhibitor or competitor. Thereafter the tissue culture plates were placed on ice, the medium was removed and the cells were washed twice with ice-cold TBS containing BSA (2 mg/ml) followed by another two washes with ice-cold TBS. Cells were

lysed by treatment with 1.0 ml of NaOH (0.3 M; 25 °C; 30 min [23]). Protein was measured by the method of Lowry et al. [29]. Degradation of ^{125}I-lipoproteins was measured as described [30].

Incubation in the presence of metabolic inhibitors was performed as follows: HepG2 cells were incubated as described above in DMEM containing 10 % LPDS. Cells were pretreated with monensin (5 μM; overnight), colchicine (10 μM; 2 h) or acrylamide (5 mM; 6 h). Bafilomycin A (250 nM), KNO$_3$ (50 mN), NaN$_3$ (1 mM), (NH$_4$)$_2$VO$_3$ (50 μM) and Na$_2$MoO$_4$ × 2 H$_2$O (250 μM) were used as ATPase inhibitors and left on cells for 60 min.

To facilitate the comparison of results obtained with ^{125}I-HDL$_3$, [^{14}C]αTocH–HDL$_3$, [^{3}H]Ch18:2–HDL$_3$ and [^{3}H]Ch–HDL$_3$, selective HDL$_3$-lipid uptake is expressed as apparent HDL$_3$ particle uptake as suggested by Pittman et al. [31]. Apparent HDL$_3$ particle uptake is expressed in terms of HDL$_3$ protein (calculated on the basis of the specific radioactivity of the corresponding HDL$_3$ preparations used) that would be necessary to deliver the observed amount of tracer. These calculations are performed to compare uptake of ^{125}I and ^{3}H tracers on the same basis.

Resecretion of αTocH

HepG2 cells were cultured on 60 mm Petri dishes (5 ml of medium; standard conditions as described above) and incubated in DMEM containing 10 % LPDS 12 h before the experiments. Cells were washed with PBS (three times) and received fresh medium containing 500 μg of [^{14}C]αTocH–HDL$_3$ protein (120 min) and cholesterol (added in 10 μl of ethanol) to down-regulate the HepG2 LDL-receptor expression to block reuptake of newly synthesized lipoproteins. The HDL$_3$-containing medium was then removed and the cells were treated with trypsin (0.05 %; 37 °C; 3 min) to remove membrane-bound HDL$_3$ particles and washed with PBS [24]. Subsequently, 3 ml of medium containing 50 μg of cholesterol/ml was left on the cells for a further 3 or 6 h. After this incubation, the medium was removed and the cellular supernatant was adjusted to a density of 1.24 g/ml by the addition of solid KBr. A discontinuous density gradient was established as follows. The density-adjusted medium was overlayered with 3 ml of 1.08 g/ml, 3 ml of 1.05 g/ml and 1 ml of PBS (10 mM) to give a final volume of 10 ml. The samples were centrifuged in a Beckman SW-41 rotor at $g_{max} = 270\,000$ (40 000 rev./min) for 24 h (15 °C). Fractions of volume 1 ml were removed from the top of the tube, transferred to preweighed Eppendorf tubes and weighed again to estimate the density of the down-loaded fractions. The radioactivity in the fractions was measured on a β-counter. The cells were lysed in 0.3 M NaOH on an orbital shaker (2 ml; 1 h; 25 °C) to measure the protein content and the remaining internalized radioactivity.

Immunoprecipitation of apoB-100

In parallel with the experiments described above, HepG2 cells were pulse-labelled with radioactive methionine, and the cellular supernatant was subjected to ultracentrifugation [32]. A 56 cm^2 Petri dish was incubated in methionine-free DMEM for 2 h, and then pulse-labelled in the presence of [^{35}S]methionine (150 μCi/dish) for 2 h. Cells were washed as described above and further incubated in the presence of methionine-containing DMEM for 3 h. The cellular supernatant was removed and subjected to ultracentrifugation in the discontinuous density gradient in an SW41 rotor exactly as described above. Fractions of volume 1 ml were down-loaded from the top of the tube and immunoprecipitated as follows. A sham precipitation was per-

formed with non-immune rabbit IgG (50 μg of IgG/ml; 3 h; 25 °C; end-over-end shaker), and then 150 μl of immunoprecipitin was added and incubated for a further 30 min as described [32,33]. The samples were then centrifuged at 15 000 **g** (13 000 rev./min) (25 °C; 3 min). ApoB-100-containing lipoproteins secreted into the medium were precipitated with a rabbit polyclonal anti-(human LDL) IgG (20 μg of IgG/fraction) and incubated as described for the sham precipitation. The pellet was washed, resuspended in 50 μl of O'Ferrals buffer [63 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 25 % (w/v) SDS] and incubated at 95 °C (5 min). The immunoprecipitated apoB-100-containing lipoproteins were separated by SDS/PAGE (3–7 % gradient gels; 25 mA/gel, 4 °C). Gels were then fixed in 30 % (w/v) trichloroacetic acid, washed in methanol containing acetic acid (5 %, v/v) and soaked in Amplify (30 min). Autoradiography of dried gels (48 h at −70 °C) was performed on Cronex Hyperfilm [34].

RESULTS

αTocH content of HDL$_3$ and LDL

Lipoproteins were analysed for αTocH content immediately after isolation and revealed a content of 1.8 ± 0.81 (HDL$_3$, five preparations) and 3.7 ± 0.28 nmol/mg of total lipoprotein mass (LDL, seven preparations). This corresponds to a molar ratio of 0.27 ± 0.12 and 9.3 ± 0.70 mol of αTocH/mol of HDL$_3$ and LDL respectively, which is comparable with other reports [13,35].

Uptake of HDL$_3$-associated αTocH by HepG2 cells

The following experiments were designed to establish whether mass transfer of HDL$_3$-associated αTocH occurred when HepG2 cells were cultured in the presence of FCS, an external
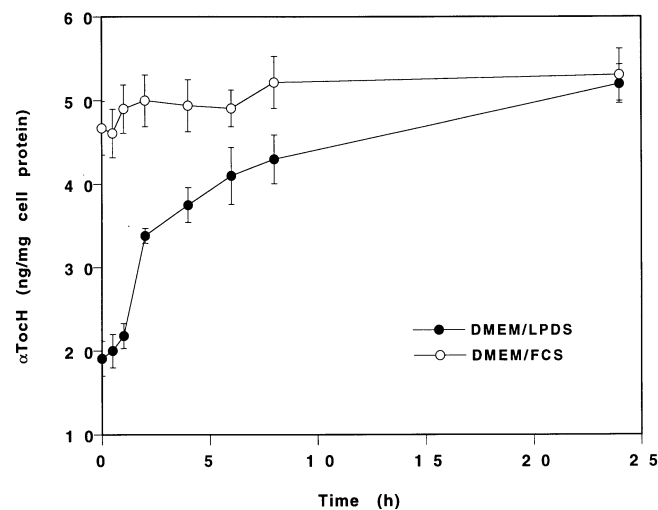
Figure 1   Uptake of unlabelled HDL$_3$-associated αTocH by HepG2 cells

Near-confluent (80–90%) HepG2 cells were cultured on 60 mm Petri dishes. Before the uptake experiment, cells were incubated in DMEM containing 10 % (v/v) FCS (○) or 10 % (v/v) LPDS (●). At time zero, the cells received 3 ml of DMEM supplemented with unlabelled HDL$_3$ (131 μg of protein; 250 ng of αTocH) and LPDS (10%, v/v). At the indicated time points, cells were washed and cellular lipids were extracted with hexane/propan-2-ol (3:2, v/v). The cellular lipid extracts were dried under argon and analysed for αTocH content by HPLC with fluorescence detection as described in the Experimental section. The cellular residues were lysed in NaOH (0.3 M) and analysed for protein content. The data are means ± S.D. from triplicate dishes from one representative experiment.
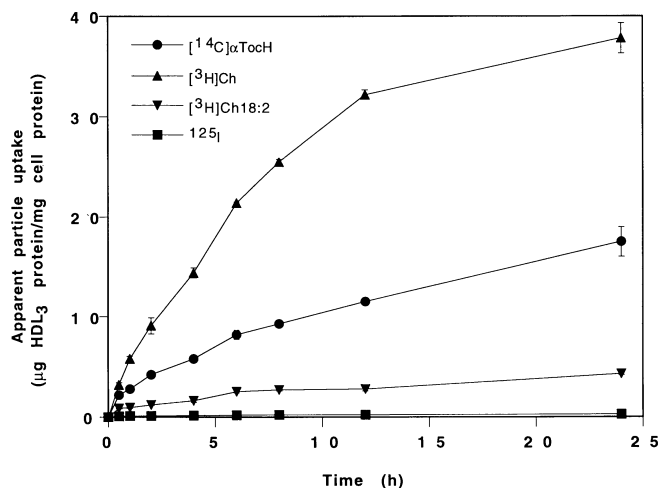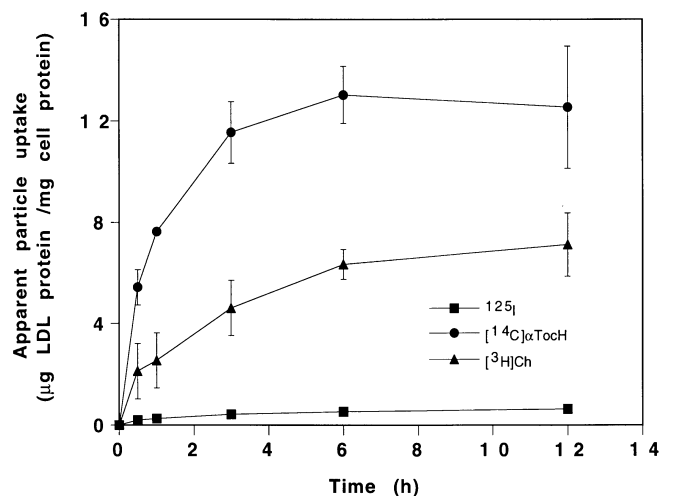
Figure 2   HDL₃ holoparticle and particle-independent lipid uptake by HepG2 cells

HepG2 cells were cultured in DMEM containing 10% (v/v) FCS on 35 mm Petri dishes until confluence. At 12 h before the uptake studies, cells were cultivated in DMEM containing 10% LPDS. Cells were washed with PBS and received fresh medium (LPDS) containing 50 μg of HDL₃ protein/ml labelled with Na^{125}I (■), [^{14}C]αTocH (●), [^{3}H]Ch18:2 (▼) or [^{3}H]Ch (▲). At the indicated time points, cells were washed twice with TBS/BSA and then twice with TBS. Aliquots of 0.3 M NaOH lysates were used to measure protein content and cell-associated radioactivity. To allow comparison of tracer uptake on the same basis, results were calculated as apparent particle uptake (i.e. the amount of protein that would account for the observed tracer uptake, if uptake were mediated by holoparticle uptake). Holoparticle uptake (^{125}I-HDL₃) represents the sum of cell-associated and degraded HDL₃. The data are means ± S.D. from triplicate dishes from one representative experiment.

Figure 3   LDL holoparticle and particle-independent lipid uptake by HepG2 cells

Cells were cultured as described in the legend to Figure 2. Preincubation was performed in DMEM containing LPDS 12 h before the uptake experiment. Cells were then incubated in the presence of 100 μg LDL protein labelled with Na^{125}I (■), [^{3}H]Ch (▲) or [^{14}C]αTocH (●). At the indicated time points, cells were washed and lysed by NaOH treatment. An aliquot was used to measure cellular protein content, and the remainder was used to determine cell-associated radioactivity. To allow comparison of tracer uptake on the same basis, results were calculated as apparent particle uptake (i.e. the amount of protein that would account for the observed tracer uptake, if uptake were mediated by holoparticle uptake). Holoparticle uptake (^{125}I-LDL) represents the sum of cell-associated and degraded LDL. The data are means ± S.D. from triplicate dishes from one representative experiment.

source of αTocH. The αTocH concentration in FCS ranged from 0.2 to 2.8 μM (four different batches analysed), a concentration that could suffice to saturate the cellular αTocH pool. Under these experimental conditions, one would expect exchange rather than net mass transfer during radiotracer experiments. To clarify this, HepG2 cells were incubated in DMEM containing either FCS (1.15 μM αTocH) or heat-inactivated LPDS devoid of αTocH. After this preconditioning period, the cells received DMEM containing LPDS and HDL₃. At the indicated time points, cells were washed and the cellular lipids were analysed for their αTocH content. Data from these experiments are shown in Figure 1. It is evident that the αTocH content of HepG2 cells precultured in DMEM containing FCS remained almost constant over the entire 24 h incubation period (46.7 and 53.1 ng/mg of cell protein at 0 and 24 h respectively). In contrast, cultivation of HepG2 cells in DMEM containing LPDS resulted in a significant reduction in cellular αTocH content when compared with DMEM/FCS conditions (19.1 ± 2.1 and 46.7 ± 3.2 ng/mg of cell protein respectively). Incubation in the presence of HDL₃ increased the cellular αTocH content from 20.0 (0.5 h) to 52.0 ng/mg of cell protein (24 h). These experiments demonstrate rapid and quantitative net mass transfer of HDL₃-associated αTocH to HepG2 cells.

To investigate αTocH-uptake mechanisms by HepG2 cells, the lipid moiety of HDL₃ was labelled with [^{14}C]αTocH and the protein moiety was labelled with Na^{125}I. These experiments were performed to compare the efficacy of αTocH uptake with that of [^{3}H]Ch and [^{3}H]Ch18:2, tracers known to be taken up via the selective uptake pathway [36]. Data from time-dependent experiments are shown in Figure 2 and are expressed in terms of apparent HDL₃ particle uptake as suggested [31]. The time-

dependent uptake of ^{125}I-HDL₃ (expressed as sum of binding, internalization and degradation) displayed saturation kinetics, the plateau value being reached within 8 h (0.21 ± 0.013 μg of HDL₃ protein/mg of cell protein). In contrast, incubation of HepG2 cells in the presence of [^{14}C]αTocH-labelled HDL₃ resulted in a constant increase in cell-associated radioactivity from 2.2 to 17.5 μg of HDL₃/mg of cell protein (2 and 24 h respectively). αTocH uptake was very efficient and exceeded that of HDL₃ holoparticle by 83-fold (17.5 μg and 0.21 μg respectively) after 24 h. For comparative reasons, cells were incubated in the presence of [^{3}H]Ch18:2-labelled HDL₃, a tracer known to be delivered to HepG2 cells via the selective uptake pathway [36]. [^{3}H]Ch18:2 uptake was also significantly higher (e.g. 6.2- and 20-fold at 6 and 24 h) than HDL₃ holoparticle uptake over the entire time course investigated. The most efficient particle-independent uptake was displayed by HDL₃-associated [^{3}H]Ch; at the 24 h time point, uptake of [^{3}H]Ch exceeded HDL₃ holoparticle uptake by 180-fold. From the results shown in Figure 2, it is evident that HDL₃-associated αTocH is delivered to HepG2 cells independently of holoparticle uptake with high efficiency.

To study further whether LDL-associated αTocH is taken up to a greater extent than LDL particles, HepG2 cells were incubated in the presence of ^{125}I-LDL or [^{14}C]αTocH-labelled LDL. As shown in Figure 3, the time-dependent uptake of αTocH exceeded LDL-particle uptake at all time points investigated. Both αTocH uptake and LDL-particle uptake were saturable processes, reaching a plateau after 6 h. At the 12 h time point, αTocH uptake exceeded ^{125}I-LDL uptake by a factor of 28 (12.5 ± 2.4 and 0.46 ± 0.014 μg/mg of cell protein respectively), demonstrating that LDL-associated αTocH also underwent particle-independent uptake in HepG2 cells. The efficacy of LDL-associated cholesterol uptake was about 11 times lower
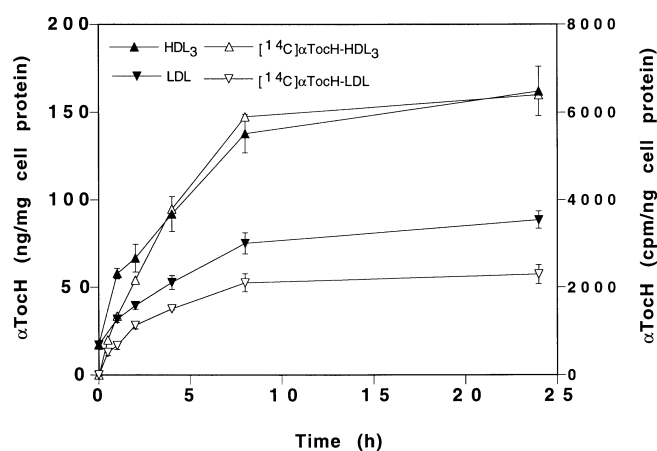
Figure 4   Comparison of αTocH mass transfer and tracer uptake

Cells were cultured as described in the legend to Figure 2 and cultured in DMEM containing LPDS 12 h before the uptake experiment. Uptake studies with radiolabelled lipoproteins were performed in six-well trays, and, for mass-transfer studies, cells were cultured in 100 mm Petri dishes. The lipoprotein concentrations were chosen on the basis of equal αTocH concentrations present as either HDL$_3$- or LDL-associated αTocH. Transfer of radiolabelled αTocH: cells were incubated in the presence of [^{14}C]αTocH-labelled HDL$_3$ (150 μg of protein; 296 ng of endogenous αTocH; specific radioactivity 177 c.p.m./ng of endogenous αTocH) and [^{14}C]αTocH-labelled LDL (15 μg of protein; 309 ng of endogenous αTocH; specific radioactivity 184 c.p.m./ng of endogenous αTocH). At the indicated time points the cells were washed and cellular radioactivity was counted in NaOH lysates. The data are means $\pm$ S.D. from triplicate dishes from one representative experiment. Transfer of endogenous αTocH ('mass transfer'): cells were incubated in the presence of unlabelled HDL$_3$ (2.2 mg of protein; 2.5 μg of endogenous αTocH) and LDL (0.20 mg of protein; 2.5 μg of endogenous αTocH) in 4 ml of DMEM containing LPDS. At the indicated time points, the cells were washed and the cellular lipids were extracted with hexane/propan-2-ol. The organic extracts were dried under argon, redissolved in 110 μl of methanol and analysed by HPLC as described in the Experimental section. The cellular residue was lysed in 3 ml of 0.3 M NaOH to measure protein content.

Table 1   Effect of metabolic inhibitors on [^{14}C]αTocH- and ^{125}I-labelled HDL$_3$ uptake by HepG2 cells

Cells were preconditioned in DMEM containing 10 % LPDS before the addition of the metabolic inhibitors. Cells then received fresh medium containing LPDS (10 %) with the indicated concentrations of the various inhibitors (added as aqueous stock solutions) for the time indicated before the addition of 100 μg of [^{14}C]αTocH–HDL$_3$ or ^{125}I-HDL$_3$ protein. After 6 h, cells were washed as described in the Experimental section, and lysed in NaOH. Aliquots of the lysate were used to measure radioactivity (800 μl) and cell protein (60 μl). Results are means $\pm$ S.D. for three determinations from one representative experiment. * $P < 0.05$ compared with control.

Inhibitor	Apparent HDL$_3$ particle uptake (% of control†)	
	[^{14}C]αTocH–HDL$_3$	^{125}I-HDL$_3$
Control	100 $\pm$ 5.2	100 $\pm$ 6.8
Monensin (5 μM; overnight)	102 $\pm$ 7.9	103 $\pm$ 5.1
Colchicine (1 μM; 120 min)	102 $\pm$ 3.4	95 $\pm$ 7.2
Acrylamide (5 mM; 360 min)	117 $\pm$ 0.5*	111 $\pm$ 4.5

† 100 % corresponds to an (apparent) particle uptake of 18.6 $\pm$ 0.97 and 0.6 $\pm$ 0.04 ng of HDL$_3$/mg of cell protein for [^{14}C]αTocH–HDL$_3$ and ^{125}I-HDL$_3$ respectively.

than uptake of HDL$_3$-associated cholesterol (Figures 2 and 3; total/particle uptake = 180 and 15.7 for HDL$_3$- and LDL-associated cholesterol respectively).

A major question arising from the results shown in Figures 2 and 3 was whether HDL$_3$- and LDL-associated αTocH are transferred at the same rates and with the same efficacy. In addition, we wanted to clarify whether uptake of [^{14}C]αTocH was by mass transfer or merely exchange. To allow a comparison of these results, HepG2 cells were incubated in the presence of HDL$_3$ and LDL adjusted for equal concentrations of radioactive and/or unlabelled (endogenous) αTocH. At the indicated time points, cells were washed and analysed for their αTocH content by liquid-scintillation counting or HPLC analysis of the cellular lipid extracts. From the data shown in Figure 4, it is apparent that the amount of [^{14}C]αTocH delivered by HDL$_3$ exceeded that transferred via LDL at all time points analysed. This effect was most pronounced after 24 h when HDL$_3$-mediated [^{14}C]αTocH transfer was approx. 3-fold higher than that mediated by LDL (6400 c.p.m. and 2300 c.p.m./mg of cell protein respectively). The same effect was observed when HepG2 cells were incubated in the presence of unlabelled HDL$_3$ or LDL. After a 24 h incubation period, cells acquired 162 ng of αTocH/mg of cell protein during an incubation in the presence of 2.5 μg of HDL$_3$-associated αTocH. In contrast, when cells were incubated in the presence of LDL containing an equal amount of αTocH, the corresponding value was only 88 ng/mg of cell protein, indicating that αTocH associated with HDL$_3$ is transferred with greater efficiency than LDL associated with αTocH (Figure 4). In

addition, these results indicate that the radiolabelled αTocH was transferred in conjunction with endogenous lipoprotein-associated αTocH.

Effects of Golgi and cytoskeletal inhibitors on the uptake of HDL$_3$-associated αTocH

To determine to what extent different metabolic inhibitors interfere with uptake of [^{14}C]αTocH–HDL$_3$ and holoparticle uptake of ^{125}I–HDL$_3$, HepG2 cells were incubated in the presence of the indicated concentrations of several metabolic inhibitors (Table 1) and the correspondingly labelled HDL$_3$ preparation. In these experiments, we used monensin (interferes with intracellular lipid transport [37]), colchicine (interferes with microtubule assembly but also disrupts Golgi membranes [38]) and acrylamide (interferes with intermediate filament action [38]). Acrylamide produced significantly enhanced αTocH uptake (1.12-fold, $P < 0.05$), but monensin and colchicine were without effect. Holoparticle uptake was not affected by any of the metabolic inhibitors used here.

Effect of ATPase inhibitors on the uptake of HDL$_3$-associated αTocH

To test whether ATPases are involved in αTocH internalization, the effect of three V-type ATPase inhibitors (bafilomycin A, NaN$_3$ and KNO$_3$ [39]) and two P-type ATPase inhibitors (molybdate and vanadate [38]) on αTocH uptake was investigated. None of these ATPase inhibitors affected the cellular capacity for αTocH uptake (Table 2).
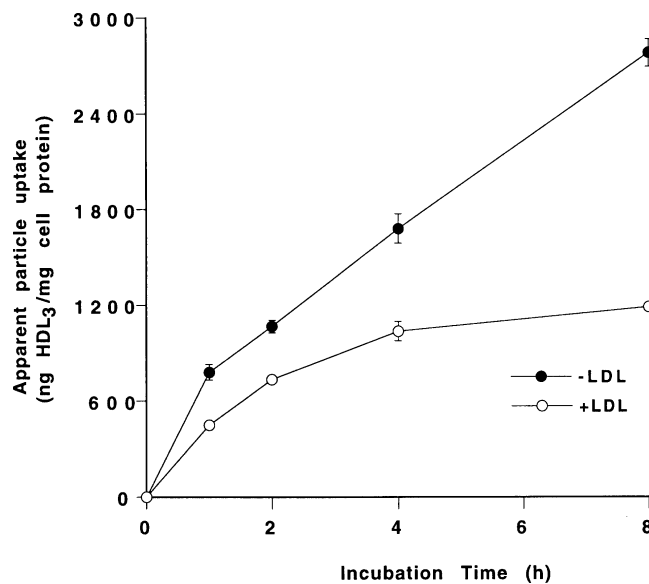
Effect of the cellular cholesterol content on the uptake of HDL$_3$-associated αTocH

Both SR-BI expression and selective uptake of HDL-associated CEs have been demonstrated to be regulated by the cellular/tissue cholesterol content [36,40]. To test the possibility that uptake of HDL-associated αTocH is also modulated by cellular cholesterol content, HepG2 cells were cultivated in the absence or presence

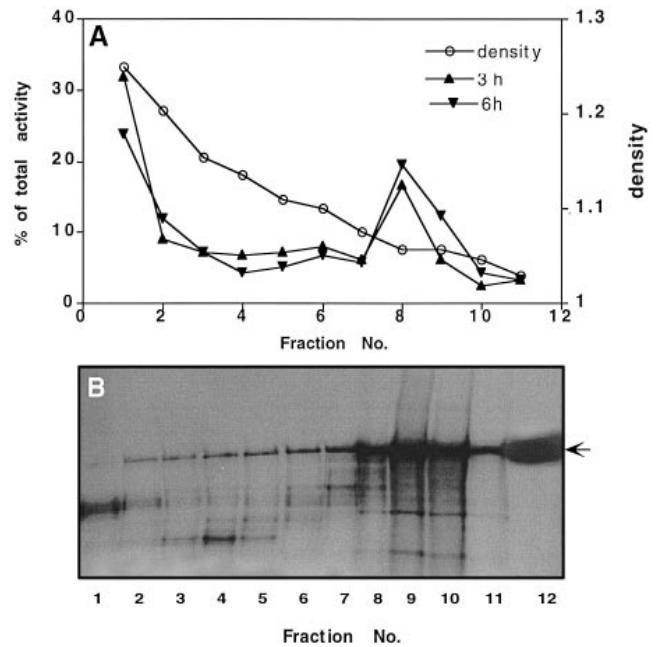**Table 2   Effect of various ATPase inhibitors on [^{14}C]αTocH uptake by HepG2 cells**

HepG2 cells were preconditioned in DMEM containing 10% LPDS (12 h) followed by a 60 min incubation in the presence of the indicated concentrations of ATPase inhibitors which were added in Hanks balanced salt solution. Cells were washed and placed in fresh Hanks balanced salt solution (containing 100 μg of [^{14}C]αTocH–HDL$_3$ protein). After 120 min of incubation, cells were washed and lysed by the addition of 1 ml of 0.3 M NaOH. Aliquots of the lysate were used to measure radioactivity (800 μl) and cell protein (60 μl). Results are means ± S.D. for three determinations from one representative experiment.

	[^{14}C]αTocH–HDL$_3$	
Inhibitor	μg/mg of cell protein	% of control
None (control)	19.4 ± 1.85	100
Bafilomycin A (250 nM)	19.7 ± 0.98	101 ± 5.1
KNO$_3$ (50 mM)	21.7 ± 0.94	112 ± 4.8
NaN$_3$ (1 mM)	20.4 ± 1.56	105 ± 8.1
(NH$_4$)$_2$VO$_3$ (50 μM)	18.8 ± 0.62	97 ± 3.2
Na$_2$MoO$_4$, 2H$_2$O (250 μM)	19.8 ± 1.66	102 ± 8.5

Figure 5   Effect of cellular cholesterol content on [^{14}C]αTocH–HDL$_3$ uptake

To modify the cellular cholesterol content, HepG2 cells were incubated on 35 mm Petri dishes in the absence (●) or presence (○) of LDL (1.5 mg of total lipoprotein mass) for 15 h in DMEM/LPDS. Thereafter the cells were washed and incubated in DMEM/LPDS containing 25 μg of [^{14}C]αTocH-labelled HDL$_3$. At the indicated time points, the cells were washed and cellular radioactivity was counted in NaOH lysates. An aliquot was used to measure cellular protein content. Results are expressed as apparent particle uptake. The data are means ± S.D. from triplicate dishes from one representative experiment.

Figure 6   αTocH delivered to HepG2 cells is resecreted in newly synthesized apoB-100-containing lipoprotein

(**A**) Confluent HepG2 cells were cultured on 60 mm Petri dishes in DMEM containing LPDS before the uptake experiments. Cells received fresh medium containing 500 μg of [^{14}C]αTocH-labelled HDL$_3$ which was left on the cells for 2 h. Cells were washed with TBS/BSA and TBS and subsequently treated with 2 ml of a 0.05% trypsin solution for 5 min at 37 °C. Then 3 ml of DMEM/10% LPDS was added to the Petri dishes and left for 3 and 6 h. The cellular supernatant was then removed, adjusted to a density of 1.24 g/ml and subjected to ultracentrifugation in a discontinuous KBr gradient in an SW-41 swing-out-bucket rotor as described in the Experimental section. The gradient fractions were down-loaded from the top of the tube and transferred to preweighed Eppendorf tubes to estimate the density of the single fractions. Radioactivity present in the down-loaded fractions was then determined. Results are means from two experiments. (**B**) In a parallel incubation, HepG2 cells were preincubated in methionine-free medium and pulse-labelled with [^{35}S]methionine (150 μCi/dish; 120 min). ApoB-100 in the gradient fractions recovered from the density gradient (lanes 1–11) was immunoprecipitated as described in the Experimental section and separated by SDS/PAGE (3–7% gels). Bands were visualized by autoradiography. The arrow indicates the position of ^{125}I-labelled apoB-100 used as a standard (lane 12).

resulted in a 2.3-fold decrease in [^{14}C]αTocH uptake, in line with observations for selective uptake of HDL-associated CEs [36].

Resecretion of internalized αTocH with newly synthesized lipoproteins

HepG2 cells are known to synthesize and secrete apoB-100-containing LDL-like particles, also termed LpB100 [32,33]. We therefore examined whether αTocH internalized by HepG2 cells independently of holoparticles is assembled with newly synthesized lipoproteins and resecreted. HepG2 cells were metabolically labelled in the presence of [^{14}C]αTocH–HDL$_3$. They were then washed and subjected to a short treatment with trypsin to remove membrane-bound lipoprotein particles. After 3 and 6 h the cellular supernatant was aspirated, subjected to density-gradient ultracentrifugation, and the radioactivity present in the single fractions was counted. These experiments indicated that resecretion of αTocH by HepG2 cells was quite efficient, i.e. within 3 h nearly 50% of the radioactivity associated with HepG2 cells after the trypsin treatment (time zero of chase) was found in the cellular supernatant (7200 ± 1136 and 15540 ± 1789 c.p.m. secreted and cell-associated radioactivity respectively

of LDL (1.5 mg of total lipoprotein mass/well). The latter incubation resulted in an increase in total cholesterol content from 28 μg (control) to 60 μg/mg of cell protein, in line with earlier reports [36]. After the incubation in the absence or presence of LDL, HepG2 cells were incubated in the presence of 25 μg of [^{14}C]αTocH-associated HDL$_3$. When cells were pre-incubated in the absence of LDL, the apparent particle uptake was 2781 ± 87 ng of HDL$_3$/mg of cell protein (Figure 5). In contrast, preincubation in the presence of LDL resulted in apparent particle uptake of 1190 ± 12 ng of HDL$_3$/mg of cell protein. Therefore a 2-fold increase in total cell cholesterol
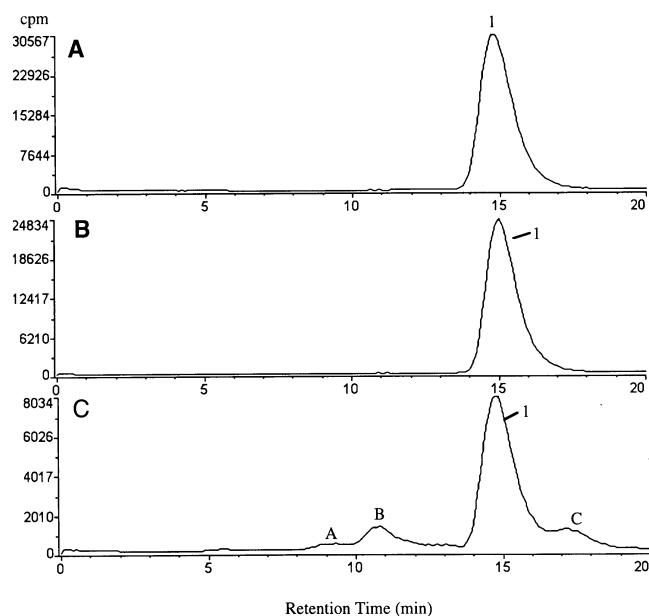
Figure 7   Radiometric HPLC analysis of cellular and resecreted [¹⁴C]αTocH

Cells were cultivated as described in Figure 6(A). Radiometric HPLC analysis of extracted [¹⁴C]αTocH (metabolites) was performed as described in the Experimental section. A 20 μl volume of the corresponding lipid extract was analysed. (**A**) Analysis of lipid extracts from [¹⁴C]αTocH–HDL₃. [¹⁴C]αTocH–HDL₃ (50 μl) was extracted in hexane/methanol (5:1, v/v; 10 ml); the hexane phase was aspirated, dried and redissolved in 400 μl of mobile phase. (**B**) Analysis of cellular lipid extracts after a 120 min pulse period in the presence of 500 μg of [¹⁴C]αTocH–HDL₃. For these experiments cells were labelled as described in Figure 6(A). Cellular lipids were extracted into hexane/propan-2-ol (3:2, v/v; 4 ml), and the extracts were dried under nitrogen and redissolved in 400 μl of mobile phase. (**C**) Analysis of [¹⁴C]αTocH resecreted into the medium during a 3 h chase period. Pulse–chase experiments were performed as described in Figure 6(A). The medium was removed and subjected to density-gradient ultracentrifugation in an SW-41 rotor as described in the Experimental section. The density fraction 1.04–1.075 g/ml was removed and extracted with hexane. The hexane phase was aspirated, dried under nitrogen and redissolved in 250 μl of mobile phase.

at time zero of chase). Analysis of the gradient fractions revealed that 40 % of the resecreted radioactivity was recovered in a density region between 1.046 and 1.074 g/ml, very similar to the buoyant density of LDL-like lipoprotein particles secreted by HepG2 cells (Figure 6A). The remaining activity was found in the top (≈ 5 %, 1.03–1.046 g/ml) and the bottom of the gradient at a density of 1.16–1.25 g/ml (40–50 %). The distribution of resecreted αTocH in the density gradient obtained after a 6 h chase was not significantly different from the results obtained after 3 h (Figure 6A). These data indicate that some of the αTocH delivered to HepG2 cells was resecreted (and assembled) with endogenously synthesized LDL-like particles.

In parallel experiments, HepG2 cells were pulse-labelled with [³⁵S]methionine to clarify whether newly synthesized apoB-100 and resecreted [¹⁴C]αTocH are co-localized in the density gradient. Cells were then chased with methionine-containing medium, and the chase medium was subjected to ultracentrifugation followed by immunoprecipitation of apoB-100 and subsequent autoradiography. Ultracentrifugation and immunoprecipitation of apoB-100 in the density fractions obtained from [³⁵S]-methionine-pulse-labelled HepG2 cells revealed that most of the apoB-100 was present in fractions 8–10 in a density interval between 1.029 and 1.069 g/ml, corresponding to the LDL-like fraction secreted by HepG2 cells (Figure 6B). The results presented in Figure 6(A) and 6(B) demonstrate that some of the

αTocH internalized by HepG2 cells was resecreted with a lipoprotein fraction floating in a density region between 1.046 and 1.074 g/ml and co-localized with most of the apoB-100 produced by endogenous synthesis.

To confirm that αTocH was still in its bioactive reduced state, both the cellular lipids and the lipids resecreted into the medium were extracted and analysed by reversed-phase HPLC with radiometric detection (Figures 7A–7C). Figure 7(A) shows the elution pattern of [¹⁴C]αTocH–HDL₃ lipid extracts and it is evident that [¹⁴C]αTocH is eluted as a single peak (peak 1, co-eluted with an unlabelled standard; not shown). To test the effect of cellular uptake on the tracer, cells were incubated for 120 min in the presence of [¹⁴C]αTocH–HDL₃, washed and the cellular lipids extracted and analysed. As can be seen from Figure 7(B), the elution pattern of the cell-associated label is identical with peak 1 shown in Figure 7(A). Another set of Petri dishes was then chased for 3 h and analysed for the secreted tracer. Figure 7(C) demonstrates the occurrence of three new peaks with shorter (peaks A and B) and slightly longer (peak C) retention times compared with [¹⁴C]αTocH. These newly occurring αTocH-derived compounds contribute approx. 15–20 % of the total peak area in Figure 7(C) after the 3 h incubation in the presence of HepG2 cells.

DISCUSSION

We present evidence that particle-independent uptake of HDL₃-associated αTocH is compatible with the concept of selective uptake described for HDL-associated CEs: (i) the efficacy of particle-independent uptake of αTocH is dependent on the size of the donor particle; (ii) cellular cholesterol content regulates the efficiency of HDL₃-associated αTocH uptake, in analogy with reports for selective uptake of HDL-associated CEs [36] and SR-BI expression [40]. The tissue distribution of αTocH in rats provides further indirect support for the assumption that HDL-associated αTocH is taken up selectively: Bjornboe and colleagues [41] reported that rat adrenal glands, one of the major sites of SR-BI expression [19] and selective HDL-CE uptake *in vivo* [42], contain the highest αTocH concentrations (on a per gram of tissue basis).

The efficacy of lipoprotein particle-independent uptake of HDL₃-associated αTocH was higher than that of Ch18:2, but lower than holoparticle-independent uptake of non-esterified cholesterol, which probably reflects the lipid polarity and/or the topographical location of the lipids within a lipoprotein particle. Most Ch18:2 is expected to reside within the hydrophobic HDL₃ core [43], whereas αTocH and cholesterol are located in the polar surface layer [20], a fact that may contribute to the greater efficiency of selective uptake, as observed in the present study. We also observed lipoprotein-particle-independent uptake of LDL-associated αTocH, in line with *in vivo* findings reported by Cohn et al. [11]. These authors have reported greater uptake of LDL-associated αTocH than lipoprotein particles by a variety of tissues in Watanabe heritable hyperlipidaemic and control rabbits.

A remarkable observation in the present study is that LDL-associated αTocH was less readily taken up than HDL-associated αTocH, a fact that should be considered during supplementation studies. This observation may be a result of the larger lipid core of the LDL particle, acting as a sink for αTocH and thereby decreasing the probability of diffusion out of the particle [44]. On the other hand, the greater curvature of the HDL₃ particle may contribute to the higher transfer rates, comparable with obser-vations reported for selective HDL-associated CE uptake [45]. The *in vivo* relevance of apoA-containing lipoproteins during

αTocH turnover has been demonstrated in patients suffering from abetalipoproteinaemia. These patients are unable to synthesize apoB-containing lipoproteins because of a defect in the microsomal triacylglycerol transfer protein [46]. When supplemented with vitamin E, some of these patients can achieve normal adipose-tissue tocopherol levels [47], which is compatible with our results.

To identify possible routes for selective uptake and/or storage of αTocH, different metabolic inhibitors were studied; most had no significant effect on uptake by HepG2 cells (Table 1). ATPase inhibitors were also without effect on selective αTocH uptake (Table 2), in line with data reported for selective uptake of HDL-associated CEs [38]. Reaven and colleagues [38] have demonstrated that N-ethylmaleimide (NEM) efficiently blocks internalization of HDL-associated fluorescently labelled CE derivatives. In accordance with a more general role of NEM as an inhibitor of selective lipid uptake, Fielding and Fielding [48] demonstrated that selective uptake of LDL-associated cholesterol is blocked by NEM. In line with these reports, we have observed a slight inhibition of αTocH uptake by HepG2 cells in the presence of NEM (D. Goti and W. Sattler, unpublished work). From our data, it appears unlikely that NEM-sensitive fusion protein, an ATPase that triggers the break-up of soluble NEM-sensitive factor attachment protein receptor complexes [49], is involved in αTocH uptake/internalization, since bafilomycin and KNO_3 failed to interfere with selective uptake of HDL-associated αTocH.

In analogy with selective uptake of HDL-associated CEs [36], the present study reveals an inverse relationship between HDL-particle-independent αTocH uptake and intracellular cholesterol content. Although this does not provide direct evidence for selective αTocH uptake via SR-BI-mediated mechanisms, our observations are at least compatible with such a concept, since SR-BI expression is regulated by the cellular cholesterol content [40]. Increased cellular αTocH concentrations (as a result of the LDL preincubation) could be an alternative explanation for the observed down-regulation of selective HDL–αTocH uptake, although this appears to be less likely. Preincubation of mouse adrenal Y-1 cells (expressing SR-BI) with cholesterol-supplemented medium resulted in significantly down-regulated HDL–αTocH uptake, comparable with effects observed in the presence of LDL (results not shown). We are at present clarifying whether the decrease in selective αTocH uptake on LDL loading is a result of increased cholesterol concentrations or a combined effect of increased intracellular cholesterol and αTocH content.

In rats, about 90 % of total αTocH is associated with the liver, adipose tissue and skeletal muscle [41]. Most of the intracellular αTocH in these animals is found in mitochondria, the endoplasmic reticulum and the Golgi apparatus [50]. The confinement of αTocH to the endoplasmic reticulum and Golgi apparatus could be important for the association with newly synthesized VLDL, since the endoplasmic reticulum is the organelle where the first steps of intracellular VLDL assembly occur [51]. Lipoprotein synthesis in HepG2 cells is a sequential process starting with the co-translational integration of apoB-100 into the membrane of the endoplasmic reticulum and subsequent transfer from the site of synthesis to the site of assembly, where it interacts with neutral lipids to form lipoprotein particles [32,33]. It is tempting to speculate that the endoplasmic reticulum may also be the intracellular compartment where αTocH is assembled with newly synthesized lipoproteins. Our pulse–chase and metabolic [^{35}S]methionine-labelling experiments demonstrate that originally HDL_3-associated αTocH, selectively taken up by HepG2 cells, can be resecreted along with newly synthesized apoB-100-containing lipoproteins, with most of the

label located in an LDL-density-like fraction and the dense region of the gradient (Figure 6A). At present, it is not clear whether the fraction of αTocH that is present in the dense region of the gradient is associated with a carrier protein. Preliminary experiments have revealed that most of this label is associated with a 60–90 kDa fraction eluted from a size-exclusion column (E. Malle and W. Sattler, unpublished work). Also the site of assembly with apoB-100-containing lipoproteins is not clear; it could be achieved by the action of the αTocH-transfer/binding protein [6]. Independently of the mechanism(s) responsible for αTocH assembly/association with newly synthesized lipoproteins, selective uptake and subsequent hepatic resecretion could contribute to the maintenance of circulating concentrations. Selective uptake of HDL-associated αTocH could also precede biliary secretion of circulating αTocH or the more polar oxidized metabolites, e.g. α-tocopherylquinone [27].

In summary, our findings demonstrate that uptake of HDL-associated αTocH occurs via a particle-independent (selective) uptake pathway; selective uptake was not inhibited by the ATPase inhibitors tested but was regulated by cellular cholesterol content, in line with findings for SR-BI expression [40]. Finally, HDL-associated αTocH taken up selectively is incorporated and resecreted with newly synthesized lipoproteins. Whether selective uptake affects αTocH-mediated signal-transduction pathways via modulation of protein kinase activity [34] remains to be elucidated.

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