Greater selective uptake by Hep G2 cells of high-density lipoprotein cholesteryl ester hydroperoxides than of unoxidized cholesteryl esters

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We have observed recently that high-density lipoproteins (HDL) are the predominant carriers of cholesteryl ester hydroperoxides (CEOOH), the major class of lipid hydroperoxides detectable at nanomolar concentrations in the plasma of healthy fasting humans. The present study investigates the effect of such very low levels of CEOOH in apolipoprotein E-free HDL₃ on lipoprotein particle metabolism and 'selective uptake' of its CE by human Hep G2 cells. Minimal oxidation with aqueous peroxy radicals had a negligible effect on the binding, internalization and degradation of ¹²⁵I-labelled HDL₃. In contrast, with an increasing degree of radical-mediated oxidation of labelled HDL₃, [³H]cholesteryl linoleate ([³H]Ch18:2) was taken up at an increasingly greater rate than were ¹²⁵I-apoproteins. When [³H]cholesteryl linoleate hydroperoxide ([³H]Ch18:2-OOH) was incorporated into unoxidized HDL₃ by exchange from donor liposomes, it was taken up at a more than 8-fold higher rate than was incorporated [³H]Ch18:2. The same degree of preferential

uptake of oxidized CE was observed when HDL₃ was used that was doubly labelled with [³H]Ch18:2-OOH and cholesteryl [¹⁴C]oleate ([¹⁴C]Ch18:1). In both situations, uptake of [³H]Ch18:2-OOH exceeded that of ¹²⁵I-apolipoprotein A-I some 40-fold. This increased selective uptake of [³H]Ch18:2-OOH from very mildly oxidized HDL₃ was accompanied by a parallel increase in the intracellular levels of labelled free cholesterol. In contrast, lipid hydroperoxides were not detectable within Hep G2 cells, suggesting efficient detoxification of CEOOH by these cells. Neither the increased selective uptake of Ch18:2-OOHs nor the levels of intracellular free cholesterol were influenced by the presence of 50 μ M chloroquine, suggesting extralysosomal hydrolysis of oxidized CEs. These results show that the selective uptake of HDL CEOOH by Hep G2 cells is more efficient than that of unoxidized CE, and support a protective role for rapid selective uptake in the removal of circulating HDL CEOOH.

INTRODUCTION

High-density lipoproteins (HDL) are anti-atherogenic, an activity thought to be due to their central role in reverse cholesterol (Ch) transport, the process by which excess Ch is removed from non-hepatic cells and transported to the liver for elimination into bile. HDL accept Ch from various cells, and some of this Ch is esterified to cholesteryl esters (CE) by the lecithin: cholesterol acyltransferase associated with HDL. The fate of the resulting CE is not entirely clear; several pathways may contribute to their eventual removal from blood.

Removal of the whole HDL particle (holoparticle uptake) is a small contributor to overall CE clearance in man, as indicated by a much shorter circulating half-life of HDL CE than of HDL apoproteins [1]. HDL may acquire apolipoprotein E (apoE), which targets the particle for increased hepatic uptake, although this pathway too appears to be of minor importance *in vivo* [2]. In contrast, cholesteryl ester transfer protein-mediated transfer of HDL CE to very-low-density lipoprotein and low-density lipoprotein (LDL) in exchange for triacylglycerol is believed to be important (reviewed in [3]). In this situation, CE are removed by hepatic clearance of these more buoyant lipoproteins. HDL CE may also be taken up directly without uptake of the lipoprotein particle, a process referred to as 'selective uptake' [4]. Selective uptake by parenchymal cells is directly coupled to secretion of Ch into bile [5]. While competing with the transfer pathway, selective uptake may be of major importance in the

clearance of HDL CE in humans, as indicated by plasma decay studies of labelled HDL injected intravenously into rabbits [6].

Selective uptake of HDL CE has been studied in various animal tissues *in vivo* [4–7], in isolated organs [8] and in various cells of different origin *in vitro* [9–11]. Included in the latter are the human-derived Hep G2 hepatoma cells [11], a cell line with morphological and functional characteristics comparable with those of liver parenchymal cells [12]. Like other human hepatoma cell lines [13], Hep G2 cells also contain binding sites for HDL [14]. However, the extent to which specific receptors are required for selective uptake of HDL CE remains unknown. In fact, there is evidence that selective uptake does not require a specific apoprotein component [15], and that interaction of cellular surface lipids with those of HDL₃ is important [16]. Selective uptake of HDL CE by Hep G2 cells is down-regulated by Ch loading of the cells, conditions under which holoparticle uptake is increased [17]. CE taken up selectively do not appear to enter the classical endosomal/lysosomal pathway [11,18].

In contrast to HDL, elevated plasma concentrations of LDL are associated with accelerated atherogenesis. There is increasing evidence that oxidatively modified LDL can lead to lipid accumulation in cells *in vitro* and in the subendothelial space *in vivo* [19]. In light of the potential importance of oxidative LDL modification we have studied the endogenous antioxidant defences surrounding and associated with LDL [20–22] using a selective and sensitive method for the detection of lipid hydroperoxides. The introduction of this assay has allowed us to

Abbreviations used: AAPH, 2,2'-azobis-(2-amidinopropane); AMVN, 2,2'-azobis-(2,4-dimethylvaleronitrile); apoE, apolipoprotein E; Ch, cholesterol; CE, cholesteryl esters; [³H]Ch18:2, [³H]cholesteryl linoleate; [¹⁴C]Ch18:1, cholesteryl [¹⁴C]oleate; CEOOH, cholesteryl ester hydroperoxides; [³H]Ch18:2-OOH, [³H]cholesteryl linoleate hydroperoxides; CL, chemiluminescence; DMEM, Dulbecco's modified Eagle's medium; HDL, high-density lipoproteins; HDL_{3ox}, mildly oxidized HDL₃; LDL, low-density lipoproteins; t_{1/2}, half-time required for lipid transfer.

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measure the very low levels (1–10 nM) of lipid hydroperoxides reported to be present in human plasma [23]. We were intrigued to find that approx. 85 % of detectable hydroperoxides of CE (CEOOH) were present in HDL, with the LDL being virtually peroxide-free [24]. Indeed, on a per lipid basis, we found that HDL CE in plasma were over 20-fold 'more oxidized' than LDL CE. Also, HDL lipids initially became more readily oxidized than those in LDL when plasma was exposed to a mild flux of radicals [24].

These findings appeared to be at odds with the putative role of oxidized LDL in atherogenesis and the anti-risk properties attributed to HDL. However, the levels of HDL CEOOH present in healthy human plasma are several orders of magnitude lower than those required to cause adverse effects [25], and thus may represent a physiological rather than a pathological situation. Since we have also observed that HDL CEOOH are rapidly removed by Hep G2 cells [24], we chose to characterize the interaction of very mildly oxidized HDL₃ with these cells, with an emphasis on testing whether oxidation of HDL CE affects their selective uptake. Our data show that HDL CEOOH are taken up more rapidly than unoxidized CE. The results suggest that, if operating *in vivo*, such efficient selective uptake of HDL CEOOH together with their rapid detoxification could represent an important antioxidant defence aimed at preventing accumulation of toxic levels of lipid hydroperoxides in the circulation and extravascular space.

MATERIALS AND METHODS

Lipoprotein isolation

Human apoE-free HDL₃ (*d* 1.12–1.21) was isolated from the plasma of adult normolipemic donors by discontinuous density gradient ultracentrifugation [26]. HDL₃ was recovered from the tubes by direct aspiration of the lipoprotein band and dialysed extensively at 4 °C against PBS (50 mM potassium phosphate, pH 7.4) purged with argon. SDS/PAGE of the HDL protein on 3.5–20 % gradient gels revealed the presence of apoA-I and apoA-II only. Buffers were treated with Chelex 100 (Bio-Rad, Richmond, CA, U.S.A.) to remove contaminating transition metals.

Labelling procedures

[³H]Cholesteryl linoleate hydroperoxide ([³H]Ch18:2-OOH) was prepared from [1,2,6,7-³H]cholesteryl linoleate ([³H]Ch18:2; DuPont–New England Nuclear, Boston, MA, U.S.A.). Briefly, 200 µCi of [³H]Ch18:2 dissolved in 200 µl toluene was oxidized at 37 °C for 5–8 h using 403 mM 2,2'-azobis-(2,4-dimethylvaleronitrile) (AMVN; Polyscience, Warrington, PA, U.S.A.) as a source of lipophilic peroxy radicals [27]. Following oxidation, the reaction mixture was dried under argon, and the residue was redissolved in 1 ml of hexane and applied to an Al₂O₃ solid phase extraction column (Activon, Sydney, Australia) preconditioned with hexane to separate oxidized from non-oxidized tracer. Unoxidized [³H]Ch18:2 was eluted with 15 ml of hexane, followed by elution of [³H]Ch18:2-OOH with 15 ml of tert-butyl methyl ether. The isolated [³H]Ch18:2-OOH was dried under argon, redissolved in methanol/*t*-butanol (1:1, v/v) and purified further by reversed phase h.p.l.c. as described below. The activity eluting between 6 and 8 min (i.e. retention time of a Ch18:2-OOH standard, prepared as described below) was collected, dried under argon and incorporated into egg yolk lecithin liposomes as described below. Usually 50–60 % of the initial amount of radioactivity subjected to AMVN oxidation was

recovered as purified [³H]Ch18:2-OOH in the appropriate h.p.l.c. fraction (specific radioactivity 71 Ci/mmol).

Labelling of HDL₃ with either [³H]Ch18:2 or [³H]Ch18:2-OOH, or double-labelling with [³H]Ch18:2-OOH and cholesteryl [1-¹⁴C]oleate ([¹⁴C]Ch18:1; DuPont–New England Nuclear), was achieved by exchange of the tracer incorporated into unilamellar donor liposomes [28]. The same lipoprotein preparation was used for the labelling with unoxidized or oxidized tracer(s) for each set of experiments. Donor liposomes were prepared by sonication of egg yolk lecithin (Sigma; 80 µg/ml in PBS) supplemented with 100 µCi of either [³H]Ch18:2 or [³H]Ch18:2-OOH. To facilitate transfer, 1 ml of HDL₃ (1.5–2 mg of protein/ml) was incubated overnight at 37 °C and with gentle shaking with 1 ml of donor liposomes (HDL₃ protein/liposome lecithin mass ratio of 45:2) together with 1 ml of lipoprotein-deficient plasma (as a source of CE transfer protein) containing 1 mM 5,5'-dithiobis-(nitrobenzoic acid) to inhibit lecithin:cholesterol acyltransferase. All experimental steps were performed under argon to avoid inadvertent oxidation. The labelled HDL₃ was then re-isolated using a two-step density gradient [29]. The resulting specific radioactivities of the isolated labelled HDL₃ were 5–15 d.p.m./ng of protein for the different lipoprotein preparations, with a difference in specific radioactivities for [³H]Ch18:2- and [³H]Ch18:2-OOH-labelled HDL₃ prepared from the same batch of lipoprotein of less than 15 %. Iodination of apoE-free HDL₃ was achieved using Na¹²⁵I with *N*-bromosuccinimide as oxidizing agent, as described by Sinn et al. [30]. The specific radioactivities of the ¹²⁵I-HDL₃ preparations obtained and used for the experiments were 300–400 c.p.m./ng of protein, with < 2 % of the radioactivity in free form.

For some experiments, solutions of [³H]Ch18:2- or ¹²⁵I-labelled HDL₃ were oxidized for 90–120 min at 37 °C in the presence of 1, 5, 20, or 50 mM 2,2'-azobis-(2-amidinopropane) (AAPH; Polyscience), an aqueous peroxy radical generator that produces radicals at a known and constant rate [27]. The reaction was terminated by removal of excess AAPH from HDL₃ by either size exclusion chromatography [using a PD-10 column (Pharmacia LKB, Bromma, Sweden) and PBS as the eluant] or extensive dialysis at 4 °C against PBS. The degree of oxidation of CE in HDL₃ was assessed by measuring the formation of CEOOH using the extremely sensitive and specific h.p.l.c. post-column chemiluminescence detection (h.p.l.c.-CL) method [31], with the modifications described in [21,29].

Reversed-phase h.p.l.c.-CL

Analysis of endogenous neutral lipids and CEOOH associated with HDL₃ was performed by h.p.l.c.-u.v./CL using a 25 cm × 0.46 cm C-18 column (Supelco, Bellefonte, PA, U.S.A.) eluted with methanol/*t*-butanol (1:1, v/v) at 1 ml/min [21,29]. To identify ³H-labelled tracers, an additional 20 µl aliquot of the redissolved hexane extract of HDL₃ was subjected to a separate h.p.l.c. run using identical chromatographic conditions as described above for unlabelled lipids, but with the effluent from the u.v. detector, set at 210 nm, collected directly in 0.5 or 1.0 ml fractions and the radioactivity in the samples determined. The ³H-labelled tracers were identified by comparing their retention times with those of known, unlabelled standards. The concentrations of purified unoxidized and oxidized lipids were calculated by comparison of peak areas with standards of known concentration [29].

Cell culture studies

Human hepatoma Hep G2 cells [17] were plated in 35 mm-diam. 12-well tissue culture plates (Costar, Cambridge, MA, U.S.A.)

and cultured under standard conditions (37 °C, 5% CO₂, 95% humidity) in Dulbecco's modified Eagle's medium (DMEM; Cytosystems, Castle Hill, Australia) containing fetal calf serum (10%, v/v; Cytosystems), streptomycin (40 µg/ml; Sigma, St. Louis, MO, U.S.A.) and penicillin (40 units/ml; Sigma). When the cells were near confluence, the monolayers were washed twice in PBS, and the cells were conditioned by overnight incubation in fresh serum-free DMEM containing BSA (2 mg/ml; Sigma) and antibiotics as mentioned above. The cells were then washed twice more in DMEM/BSA, and the HDL₃ binding and metabolism assays were initiated. Where used, cells were preincubated for 2 h at 37 °C in DMEM/BSA in the presence of the metabolic inhibitor chloroquine prior to washing and lipoprotein metabolism studies [32]. The cell protein content was about 700 µg/35 mm well.

For lipoprotein binding, internalization and metabolism, cells were incubated for 4 h under standard conditions with radiolabelled HDL₃ at the indicated concentration and temperature in the presence or absence of excess (2 mg/ml) unlabelled HDL₃, and, where noted, the appropriate amount of metabolic inhibitor. Following incubation, the tissue culture plates were placed on ice, the medium was removed and the cells were washed twice with cold PBS/BSA followed by another two washes with cold PBS. To release cell-membrane-bound HDL₃, cells were incubated at 37 °C for 10 min in the presence of trypsin (0.05%; Cytosystems), containing 0.02% EDTA. The trypsin-releasable fraction is referred to as 'bound' HDL₃. Following trypsinization, cells were again washed (twice in PBS/BSA followed by twice in PBS) and then lysed by treatment with 1.5 ml of 0.1 M NaOH at room temperature for at least 30 min to determine both the non-trypsin-releasable or 'internalized' fraction and the cell protein present in the lysate [17].

Degradation of ¹²⁵I-HDL₃ by Hep G2 cells was estimated by measuring the trichloroacetic acid-soluble activity in the medium after precipitation of free iodide with AgNO₃ [33].

To assess the ability of Hep G2 cells to metabolize CE of increasingly oxidized HDL₃ particles, cells were incubated at 37 °C for 4 h with 50 µg of [³H]Ch18:2-labelled, AAPH-oxidized HDL₃ (see above) in the presence or absence of 50 µM chloroquine, which inhibits lysosomal hydrolysis of CE. Following incubation, cells were washed as described above and the cellular lipids were extracted with 2 × 1 ml of hexane/propan-2-ol (3:2, v/v) for 30 min at room temperature on an orbital shaker. The organic extracts were removed, dried under argon and redissolved in 200 µl of the extraction solvent. The dry cell residues were used to determine the cell protein. Extracted lipids were then separated on plastic silica sheets (Polygram Sil G; Marchery-Nagel, Dueren, Germany) using hexane/diethyl-ether/acetic acid (45:5:1, by vol.) as the solvent system. The bands corresponding to standards of free Ch, Ch18:2 and Ch18:2-OOH were visualized with iodine vapour, cut out, and the radioactivity counted. Since the cell extracts contained only very small amounts of oxidized CE which were not visible after staining, these lipids were collected as a relatively broad band. This analysis procedure does not discriminate between Ch18:2-OOH and Ch18:2-OH.

Preparation of Fab fragments

Fab fragments were prepared from polyclonal rabbit anti-apoA-I IgG (kindly provided by Drs. G. M. Kostner and E. Malle, Department of Medical Biochemistry, University of Graz, Austria) using Sepharose-immobilized papain according to the supplier's directions (Pierce, Rockford, IL, U.S.A.). Fab fragments were separated from Fc fragments and undigested IgG

by affinity chromatography on Protein-A-Sepharose. The unbound fraction (containing the Fab fragments) was analysed by SDS/PAGE (7% polyacrylamide) and found to be free of IgG.

All protein concentrations were determined by the method of Lowry et al. [34].

RESULTS

Formation of very mildly oxidized HDL₃

Controlled oxidation of ¹²⁵I-labelled HDL₃ was achieved by incubation of the lipoprotein at 37 °C with AAPH [27]. In a typical experiment, incubation of HDL₃ with 1 mM AAPH for 90 min at 37 °C resulted in Ch18:2-OOH/Ch18:2 ratios of (3.5–4) × 10⁻³. Thus, in mildly oxidized HDL₃ (HDL_{3ox}), one out of four or five lipoprotein particles carries one molecule of Ch18:2-OOH (assuming that each HDL₃ particle on average contains 60 molecules of CE).

Effect of very mild oxidation on HDL₃ apoprotein uptake by Hep G2 cells

Very mild oxidation of HDL₃ did not cause detectable differences in the specific or non-specific binding of the lipoprotein particle of Hep G2 cells at 4 °C (results not shown). Exposure of Hep G2 cells of HDL_{3ox} also did not influence cell viability or growth, as determined by [³H]thymidine incorporation (results not shown). Furthermore, similar results for ¹²⁵I-HDL₃ and ¹²⁵I-HDL_{3ox} were obtained when binding was carried out at 37 °C (Figure 1; specific binding only is shown), indicating that the metabolic fate of HDL₃ in Hep G2 cells is not altered upon very mild oxidation. Binding and internalization tended to level off at higher HDL₃ concentrations (Figures 1a and 1b), while the amount of degraded HDL increased linearly over the concentration range investigated (Figure 1c). These results are in good agreement with reports by others [11,17,35,36]. Since apoA-I might be most important in facilitating binding of HDL₃ to Hep G2 cells [37], we also tested binding of ¹²⁵I-HDL₃ and ¹²⁵I-HDL_{3ox} to HepG2 cells in the presence of intact anti-apoA-I IgG and Fab fragments. Intact anti-apoA-I IgG inhibited binding by up to 40%, and the corresponding Fab fragments reduced binding by up to 60%. No difference was observed between ¹²⁵I-HDL₃ and ¹²⁵I-HDL_{3ox} (results not shown).

Together, our studies with the ¹²⁵I-labelled lipoproteins show that very mild oxidation of HDL₃ does not affect its binding, internalization and degradation by HepG2 cells.

Uptake of HDL [³H]Ch18:2 and [³H]Ch18:2-OOH by Hep G2 cells

Since we have observed that HDL Ch18:2-OOH is rapidly removed when the lipoprotein is incubated with Hep G2 cells [24], we decided to compare the efficacy of selective uptake of unoxidized and oxidized HDL CE by these cells. One possible mechanism by which HDL could acquire preformed lipid hydroperoxides is uptake from cell membranes and/or other lipoproteins. We therefore first introduced either [³H]Ch18:2 or [³H]Ch18:2-OOH into HDL₃. Figure 2(a) shows the h.p.l.c. traces of labelled HDL₃ hexane extracts. [³H]Ch18:2 eluted as a single, sharp peak with a retention time identical to that of endogenous Ch18:2 of non-labelled HDL₃. Separation of the extracted [³H]Ch18:2-OOH-labelled HDL₃ (Figure 2a) revealed that about 95% of the activity eluted as a single peak with a retention time very close to that of unlabelled Ch18:2-OOH (Figure 2b). This provides good evidence that the majority of the HDL-associated tracer was in the form of (original) [³H]Ch18:2-OOH. The remaining 5% of activity eluted as a separate, more polar peak in front of [³H]Ch18:2-OOH. The labelled HDL₃

Figure 1 Binding (a), internalization (b) and degradation (c) of ^{125}I -HDL₃ and ^{125}I -HDL_{3ox} by Hep G2 cells

Hep G2 cells were preincubated at 37 °C for 20 h in DMEM/BSA. They then received 1 ml of fresh DMEM/BSA containing the indicated amounts of ^{125}I -HDL₃ or ^{125}I -HDL_{3ox} in the presence or absence of excess unlabelled HDL₃ (2 mg/ml), and were incubated at 37 °C for 4 h. Following incubation, the cells were washed and the trypsin-releasable (bound), non-trypsin-releasable (internalized) and trichloroacetic acid-soluble (degraded) activities were determined as described in the Materials and methods section. Specific data are shown only. Values for unspecific binding (at 200 μg/ml HDL₃) were 71, 38 and 65% (of the total) for ^{125}I -HDL₃ binding, internalization and degradation respectively, while the corresponding values for ^{125}I -HDL_{3ox} were 69, 46 and 64% (means of two independent experiments).

Figure 2 Reversed-phase h.p.l.c. of HDL₃, $[\text{^3H}]\text{Ch18:2}$ and $[\text{^3H}]\text{Ch18:2-OOH}$ (a) and of a non-labelled Ch18:2-OOH standard (b)

H.p.l.c. conditions are described in the Materials and methods section. To determine the elution profile of the radiolabelled Ch18:2, the effluent was collected immediately in 0.5 or 1 ml fractions post-u.v.-detector, and the recovered activity was determined by scintillation counting. The results in (a) are expressed as percentages of the total radioactivity eluted during the 30 min h.p.l.c. run. The Ch18:2-OOH standard (b) was prepared as described in the Materials and methods section. Peak 1 is Ch18:2-OOH.

contained about one $[\text{^3H}]\text{Ch18:2-OOH}$ molecule per 110–330 HDL₃ particles (as determined by h.p.l.c. from the ratios of HDL₃ $[\text{^3H}]\text{Ch18:2-OOH}$ to endogenous Ch18:2 of 5×10^{-5} – 1.7×10^{-4}). This variation was derived from differences in the specific radioactivity of the different radiolabelled preparations used.

We next compared binding and internalization of $[\text{^3H}]\text{Ch18:2}$ -labelled with $[\text{^3H}]\text{Ch18:2-OOH}$ -labelled HDL₃ by Hep G2 cells. Unoxidized ^{125}I -labelled HDL₃ served as a control for lipoprotein particle uptake. $[\text{^3H}]\text{Ch18:2-OOH}$ -labelled HDL₃ displayed slightly (up to 3-fold) enhanced binding when compared with $[\text{^3H}]\text{Ch18:2}$ -labelled HDL₃, which itself was almost indistinguishable from binding of ^{125}I -HDL₃ (Figure 3a). As suggested [11], results were expressed in terms of 'apparent particle uptake', i.e. the amount of HDL protein taken up as calculated from the amount of lipid tracer associated with cells, assuming that holoparticle uptake only takes place. The values for maximal specific binding at 200 μg/ml ligand were 199 ± 18 , 240 ± 23 and 323 ± 17 ng of protein/mg of cell protein for ^{125}I -HDL₃, $[\text{^3H}]\text{Ch18:2-HDL}_3$ and $[\text{^3H}]\text{Ch18:2-OOH-HDL}_3$ respectively (mean values $\pm$ S.D. for three independent experiments using three different lipoprotein preparations).

Figure 3 Binding (a) and internalization (b) of ¹²⁵I-, [³H]Ch18:2- and [³H]Ch18:2-OOH-labelled HDL₃ by HepG2 cells

Cells were incubated with the indicated amounts of different ligands as described in Figure 1. Values shown represent means $\pm$ S.D. of three independent experiments performed in duplicate using different preparations of labelled HDL₃. Cellular association of the tracers is expressed as apparent particle association, as described [11]. Specific data only are shown. Values for unspecific binding were $72.0 \pm 4.3\%$ (for [³H]Ch18:2) and $68.8 \pm 5.8\%$ ([³H]Ch18:2-OOH), while those for unspecific internalization were $38 \pm 2.7\%$ ([³H]Ch18:2) and $27 \pm 1.9\%$ ([³H]Ch18:2-OOH) of total values (means $\pm$ S.D. of three independent experiments; 200 μg/ml labelled HDL₃).

Hep G2 cells internalized significantly more [³H]Ch18:2 than ¹²⁵I-apoA-I from labelled HDL₃ (Figure 3b). The maximal (specific) apparent particle uptake was 0.87 ± 0.10 and 0.17 ± 0.02 μg of protein per mg of cell protein for [³H]Ch18:2 and ¹²⁵I-HDL₃ respectively. This 5-fold higher rate of uptake of HDL CE compared with HDL particles is well within the published range (2.5–10-fold) for selective uptake of HDL by Hep G2 cells [11,17,36]. Most strikingly, internalization of HDL₃ [³H]Ch18:2-OOH was up to 8.5-fold higher than that of unoxidized [³H]Ch18:2, thereby exceeding the fractional rate of ¹²⁵I-HDL₃ uptake some 43 times (Figure 3b). To our knowledge, this represents the highest value reported for selective uptake of HDL CE. While exposure of Hep G2 cells to [³H]Ch18:2-OOH-HDL₃ resulted in rapid accumulation of radiolabel with the cells, we consistently failed to detect CEOOH in the organic extracts of cells (results not shown).

To test whether the presence of oxidized Ch18:2 in HDL₃ influenced the selective uptake of its unoxidized Ch18:2, and vice versa, we carried out binding and internalization experiments

Figure 4 Time-dependent association of [³H]Ch18:2-labelled and [³H]Ch18:2-OOH-labelled HDL₃ with HepG2 cells (a) and kinetic analysis of the binding data (b)

Cells were preincubated in DMEM/BSA 20 h prior to the experiment. After this preincubation period, cells received fresh DMEM/BSA containing 50 μg/ml [³H]Ch18:2-labelled or [³H]Ch18:2-OOH-labelled HDL₃. At the indicated time points the cells were washed and the cell-associated radioactivity was determined as described in the Materials and methods section. Values in (a) are expressed as specific apparent particle association, as described [11]. Values in (b) were calculated from the data shown in (a) and are expressed as the time-dependent fraction of the label remaining with the lipoprotein, i.e. $\ln (CE_t/CE_0 \times 100)$. Abbreviations: CE₀, concentration of Ch18:2 or Ch18:2-OOH present at time zero (= 50 μg/ml); CE_t, remaining Ch18:2 or Ch18:2-OOH in the supernatant. Linear regression analysis revealed $r = 0.98$ for both Ch18:2 and Ch18:2-OOH.

with HDL₃ labelled either with both [³H]Ch18:2-OOH and [¹⁴C]Ch18:1, or with the appropriate amounts of [³H]Ch18:2-OOH or [¹⁴C]Ch18:1 alone. No difference in the relative extent of selective uptake of unoxidized and oxidized CE was observed whether double- or single-labelled HDL₃ was used (results not shown), suggesting that CE and Ch18:2-OOH were taken up independently of each other by Hep G2 cells.

Figure 4(a) shows the time-dependent uptake by Hep G2 cells of 50 μg/ml HDL₃ labelled with either [³H]Ch18:2 or [³H]Ch18:2-OOH. Association of [³H]Ch18:2-OOH with the cells was characterized by a very rapid initial phase: within the first 7 min of incubation, Hep G2 cells acquired about two times more [³H]Ch18:2-OOH than the amount of [³H]Ch18:2 acquired over the entire period of 180 min. At the end of the incubation period the apparent uptake of HDL₃ [³H]Ch18:2-OOH exceeded that of [³H]Ch18:2 by a factor of 8.6 (2.85 μg versus 0.33 μg of HDL₃ protein/mg of cell protein), in good agreement with the data presented in Figure 3(b). Analysis of the data shown in

Table 1 Characterization of control and AAPH-oxidized unlabelled and [^3H]Ch18:2-labelled HDL₃

Native and [^3H]Ch18:2-labelled HDL₃ was incubated at 37 °C for 120 min in the presence of the indicated concentration of the radical generator AAPH before being extracted and its neutral lipids analysed for Ch18:2 and Ch18:2-OOH as described in the Materials and methods section. Lipid concentrations were calculated by peak area comparison with standards of known concentration. The concentration of the tracers in either the Ch18:2 or Ch18:2-OOH fraction was calculated from the radioactivity recovered in the appropriate peak eluting from the h.p.l.c. and the specific radioactivity of the tracer used (i.e. 71 Ci/mmol). The data represent mean values of duplicate determinations.

AAPH (mM)	Endogenous CE (nmol/mg of protein)			Incorporated [^3H]CE (nmol/mg of protein)		
	Ch18:2	Ch18:2-OOH	Ch18:2-OOH/Ch18:2 ratio	Ch18:2	Ch18:2-OOH	Ch18:2-OOH/Ch18:2 ratio
0 (control)	375.2	0.051	1.4×10^{-4}	0.099	0.007	7.1×10^{-2}
1	401.3	4.31	1.7×10^{-2}	0.109	0.010	9.2×10^{-2}
5	340.1	24.2	7.1×10^{-2}	0.095	0.020	2.1×10^{-1}
20	198.6	31.1	1.7×10^{-1}	0.059	0.038	6.4×10^{-1}
50	140.6	38.1	2.7×10^{-1}	0.044	0.045	1.027

Figure 5 Effect of AAPH-mediated oxidation of HDL₃ on selective uptake of its Ch18:2 and particle uptake by HepG2 cells

[^3H]Ch18:2 and [^{125}I]HDL₃ were oxidized to various degrees by AAPH, and aliquots of the different lipoprotein solutions were analysed for endogenous lipids and incorporated [^3H]Ch18:2 by reversed-phase h.p.l.c. as described in the Materials and methods section. The remaining lipoprotein was used for cell experiments. Cells were preincubated in DMEM/BSA as described in the legend to Figure 4 before receiving fresh DMEM/BSA containing 50 $\mu\text{g}/\text{ml}$ [^{125}I]HDL₃ and [^3H]HDL₃ with the determined [^3H]Ch18:2-OOH/[^3H]Ch18:2 ratios. Total cell-associated radioactivity was determined following a 4 h incubation at 37 °C as described in the Materials and methods section. Selective uptake was calculated by subtracting particle uptake from apparent particle uptake.

Figure 4(a) revealed that the uptake of both HDL₃ [^3H]Ch18:2 and [^3H]Ch18:2-OOH fitted first-order reactions, with rate constants of $5.2 \times 10^{-7} \text{ s}^{-1}$ and $4.7 \times 10^{-6} \text{ s}^{-1}$ respectively (Figure 4b); and $r = 0.98$ for both Ch18:2 and Ch18:2-OOH.

Uptake of CE from unoxidized and radical-oxidized labelled HDL₃ by Hep G2 cells

In addition to the above-mentioned transfer of preformed lipid hydroperoxides, the presence of CEOOH in HDL *in vivo* may be the result of direct oxidation of the lipoprotein particle by as yet undefined mechanisms. To examine whether Ch18:2-OOHs formed in HDL₃ *in situ* were also selectively taken up by Hep G2 cells, possibly to an extent greater than that of unoxidized Ch18:2, we exposed [^3H]Ch18:2-labelled HDL₃ to various controlled fluxes of aqueous peroxy radicals. The effect of radical oxidation of HDL₃ on its endogenous Ch18:2 and incorporated [^3H]Ch18:2 is shown in Table 1. As can be seen, our 'control' HDL₃ contained significant amounts of Ch18:2-OOH, most probably formed, at least in part, during exchange and re-isolation procedures. As expected, oxidation of HDL₃ with increasing concentrations of AAPH resulted in increasing Ch18:2-OOH/Ch18:2 ratios in both unlabelled and labelled HDL₃.

Figure 5 shows the effect of such an increasing degree of HDL₃ oxidation on lipoprotein particle and selective uptake of Ch18:2

Table 2 Apparent particle uptake of unoxidized and oxidized [^3H]Ch18:2-labelled HDL₃ and intracellular fate of Ch18:2 in the absence and presence of the lysosomal inhibitor chloroquine

[^3H]Ch18:2-labelled HDL₃ was oxidized at 37 °C for 120 min in the presence of the AAPH concentrations indicated in Table 1. Following removal of AAPH, oxidized [^3H]HDL₃ (50 μg) was added to Hep G2 cells that had been pre-cultured in the absence or presence of chloroquine (50 μM). The results (mean values of duplicate determinations) are presented as apparent particle uptake as described in the legend to Figure 3. To analyse intracellular free Ch, cells were extracted and the organic extracts separated on precoated plastic t.l.c. plates as described in the Materials and methods section. After development with iodine vapour, the bands corresponding to free Ch ($R_f = 0.076$), Ch18:2-OOH ($R_f = 0.314$) and Ch18:2 ($R_f = 0.912$) were cut out and the radioactivity present was determined. Calculation of the absolute concentrations of free Ch is based on the specific radioactivity of the tracer of 71 Ci/mmol.

^3H]CEOOH/ [^3H]CE ratio	Apparent particle uptake (ng of HDL ₃ /mg of cell protein)		Intracellular free Ch (FC) (pg/mg of cell protein)		Isotope ratio FC/[FC + CEOOH + CE]	
	Control	+ Chloroquine	Control	+ Chloroquine	Control	+ Chloroquine
0.071	387.9	359.0	9.6	8.9	0.562	0.539
0.092	439.2	381.4	11.9	11.7	0.602	0.633
0.213	843.6	629.6	24.1	23.9	0.748	0.774
0.625	1090.9	958.8	37.7	40.3	0.792	0.785
1.020	1150.0	916.6	28.1	26.8	0.783	0.743

by Hep G2 cells. Holoparticle uptake was little affected by the degree of HDL₃ oxidation. Apparent particle uptake of control [³H]Ch18:2-labelled HDL₃ exceeded holoparticle uptake by a factor of 2.7 (Figure 5). The degree of selective uptake of [³H]Ch18:2 initially increased rapidly with an increasing degree of HDL₃ oxidation, and then a plateau was observed when about 50 % of the tracer was present in the oxidized form. Thus, while selective uptake is greater for HDL₃ labelled with [³H]Ch18:2-OOH by exchange from donor liposomes (Figure 3b), it is evident from our data that *in situ*-generated HDL₃ [³H]Ch18:2-OOH is also selectively taken up by Hep G2 cells, and to an extent exceeding the uptake of unoxidized [³H]Ch18:2.

To assess whether HDL₃ Ch18:2-OOHs delivered to Hep G2 cells were hydrolysed lysosomally or not, [³H]Ch18:2-labelled HDL₃, oxidized to various degrees, was incubated with the cells in the absence or presence of 50 μ M chloroquine. The results show (Table 2) that the increase in apparent particle uptake observed with increasingly oxidized HDL₃ was consistently lower (10–25 %) in the presence of chloroquine than in its absence. A partial inhibition by chloroquine of selective uptake of HDL CE has also been reported previously [11,38]. In contrast, following a 4 h incubation, intracellular amounts of free (hydrolysed) [³H]Ch were nearly identical whether chloroquine was present or not. The relative contribution of free to total (free plus esterified) [³H]Ch increased by up to 44 % with increasing degree of oxidation of the lipoprotein. These findings suggest that, the more oxidized HDL₃ is, the more readily its CEOOH become selectively taken up and hydrolysed by Hep G2 cells. Hydrolysis of oxidized and unoxidized CE appeared to take place outside the lysosomes, as chloroquine did not affect this activity (Table 2).

DISCUSSION

We have previously identified HDL as the main carrier of the very low amounts of CEOOH detectable in the plasma of human healthy donors [24]. The results obtained in the present study show that the presence of Ch18:2-OOH at up to 1 molecule of oxidized lipid per 4–5 HDL₃ particles does not cause any gross alterations in lipoprotein binding and metabolism by Hep G2 cells. However, Ch18:2-OOH of such very mildly oxidized HDL₃ are taken up by these cells in a process that is largely insensitive to chloroquine, and to an extent exceeding that of unoxidized CE and particle uptake. Such enhanced selective uptake of Ch18:2-OOH was observed whether oxidized lipids in HDL₃ resulted from *in situ* oxidation of intact lipoprotein or from transfer of pre-existing CEOOH to the lipoprotein, suggesting that this process is operative independent of their source and mechanism of origin. The physiological relevance of our *in vitro* results will have to be determined by appropriate *in vivo* experiments.

Two lines of evidence are presented in support of preferential selective uptake of HDL₃ Ch18:2-OOH over unoxidized Ch18:2. Firstly, the increasing rate of selective uptake of ³H-labelled lipid tracer corresponded closely with the increase in the Ch18:2-OOH/Ch18:2 ratio during (peroxyl)radical-mediated oxidation of [³H]Ch18:2-labelled HDL₃. The increase in the amount of cell-associated tracer was not due to increased lipoprotein particle binding and uptake. Secondly, when oxidized core lipids were transferred to HDL₃ from donor liposomes, enhanced selective uptake of Ch18:2-OOH over Ch18:2 was observed whether single- or double-labelled lipoproteins were used.

While selective uptake of HDL CE has been reported for many different cells, tissues and animals, relatively little is known about the precise molecular basis of the mechanism involved. The

uptake of oxidized HDL core lipids by Hep G2 or other cells has not been investigated previously. Green and Pittman [39] have shown that selective uptake of HDL CE by Y1-BS1 adrenocortical mouse tumour cells shows some preference for shorter and more unsaturated acyl chains; cellular uptake of ³H-labelled C₁₈ CE increased with an increasing number of C-C double bonds. They argued that the rate of selective uptake may be proportional to the solubility of the CE in the cell plasma membrane, in line with studies by Smaby and Brockman [40]. We cannot rule out the possibility that CEOOHs are more soluble in membranes than are CE. However, in the light of our findings, it is tempting to speculate that the increased selective uptake of unsaturated CE observed by Green and Pittman [39] was, at least in part, due to the presence of small amounts of CEOOH in their tracer and/or labelled HDL preparation. The levels of contaminating Ch18:2-OOH would be expected to be proportional to the degree of unsaturation of the lipid label. We noted that, although all possible precautions were taken to avoid inadvertent oxidation during our experiments, the tracer in our control HDL₃ contained significantly higher amounts of Ch18:2-OOH than did freshly isolated HDL. Indeed, the data in Table 1 and Figure 2(a) show that the different [³H]HDL preparations contained between approx. 5 and 7 % of the total radioactivity as [³H]Ch18:2-OOH.

At present it is not clear why selective uptake of exogenously incorporated HDL₃ [³H]Ch18:2-OOH exceeded that of [³H]Ch18:2-OOH generated by *in situ* oxidation of the lipoprotein. The two labelling procedures may well result in different topographical locations of the lipid tracer in the lipoprotein, a parameter expected to be important in determining the efficacy of selective uptake. We anticipate that radical-mediated peroxidation of HDL CE predominantly takes place within the core of the lipoprotein, with some of the CEOOH formed possibly translocating to the monolayer on the lipoprotein surface. In contrast, one could envisage that most of the small amounts of [³H]Ch18:2-OOH transferred to HDL from liposomes remains located in the surface monolayer. Unoxidized CE can account for up to 1 % (w/w) of lipoprotein monolayers [41] and 2–4 mol% of the lipids in membrane bilayers [42], and this relative contribution is likely to be at least as high for the more polar CEOOH. Thus it is reasonable to assume that preferential surface location of the transferred CEOOH could contribute to their relatively higher rate of selective uptake by Hep G2 cells.

In addition to their location, the polarity of the lipids is also likely to influence the rate of selective uptake. Assuming that the initial transfer of CE from the lipoprotein to the plasma membrane of Hep G2 cells is not mediated by and/or dependent on proteins [35,43,44], selective uptake may require diffusion of the CE from HDL across the water phase, as suggested for Ch during its efflux from cellular plasma membranes to HDL [45]. Hydration of the lipophilic CE could represent an important, perhaps rate-limiting, step in selective uptake. By analogy to the transfer of the lipophilic bilirubin from albumin to synthetic phospholipid membranes [46], one could imagine that the overall transfer rate of CE from a donor particle (P_d, i.e. HDL) to an acceptor particle (P_a, i.e. Hep G2 cell membrane) is determined by the initial hydration reaction (reaction 1), that is followed by a much faster transfer of the hydrated CE into the cell membrane (reaction 2):

If hydration is indeed involved in selective uptake, one could expect [47] a higher rate constant for the hydration (and hence

transfer) of Ch18:2-OOH than for the more hydrophobic CE. Our observed rate constants of $5.2 \times 10^{-7} \text{ s}^{-1}$ and $4.7 \times 10^{-6} \text{ s}^{-1}$ for the transfer of [^3H]Ch18:2 and [^3H]Ch18:2-OOH respectively (Figure 4b) support this hypothesis. The half-times ($t_{1/2}$) for transfer, calculated from the rate constants, are 370 h ([^3H]Ch18:2) and 41 h ([^3H]Ch18:2-OOH). The latter value is within the range of $t_{1/2}$ values estimated for Ch efflux from the slowly exchanging pool of J774 cells [48]. The presence of a polar (hydroxy) group in Ch enhances its transfer some 60 fold [49].

Independent of the precise molecular mechanisms of transfer involved in the selective uptake of HDL₃ Ch18:2-OOH by Hep G2 cells, once inside the cells, oxidized CE, like unoxidized CE, become hydrolysed in a compartment largely unaffected by chloroquine. Hydrolysis of Ch18:2-OOH may represent the initial step in the cellular detoxification of this oxidized lipid, with the produced non-esterified fatty acid hydroperoxide serving as a well known substrate for the highly efficient cellular antioxidant enzyme glutathione peroxidase (EC 1.11.1.9). Alternatively, CEOOH themselves could serve as substrates for phospholipid hydroperoxide glutathione peroxidase which is present in Hep G2 cells [50], or for any other presently unknown peroxidase/reductase. In any case, it is clear from our data that selectively taken-up HDL₃ Ch18:2-OOH is detoxified efficiently by Hep G2 cells. Rapid removal of HDL CEOOH may not be a unique feature of hepatocytes. Indeed, preliminary results suggest that human peripheral blood mononuclear cells (but not human umbilical vein endothelial cells) are also able to efficiently remove HDL₃-associated CEOOH in excess of CE from the culture medium (W. Sattler and R. Stocker, unpublished work). If occurring *in vivo* this (hepatic) detoxification route may represent an important antioxidant defence mechanism, preventing the accumulation of lipid hydroperoxides in HDL and possibly other lipoproteins.

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