Low- and High-Density Lipoprotein Metabolism in HepG2 Cells Expressing Various Levels of Apolipoprotein E[†]

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ABSTRACT: To determine the importance of hepatic apolipoprotein (apo) E in lipoprotein metabolism, HepG2 cells were transfected with a constitutive expression vector (pRc/CMV) containing either the complete or the first 474 base pairs of the human apoE cDNA inserted in an antisense orientation, for apoE gene inactivation, or the full-length human apoE cDNA inserted in a sense orientation for overexpression of apoE. Stable transformants were obtained that expressed 15, 24, 226, and 287% the apoE level of control HepG2 cells. The metabolism of low-density lipoprotein (LDL) and high-density lipoprotein-3 (HDL_3), two lipoprotein classes following both holoparticle and cholesteryl esters (CE)-selective uptake pathways, was compared between all these cells. LDL-protein degradation, an indicator of the holoparticle uptake, was greater in low apoE expressing cells than in control or high expressing cells, while HDL_3-protein degradation paralleled the apoE levels of the cells ($r^2 = 0.989$). LDL- and HDL_3-protein association was higher in low apoE expressing cells compared to control cells. In opposition, LDL- and HDL_3-CE association was not different from control cells in low apoE expressing cells but rose in high apoE expressing cells. In consequence, the CE-selective uptake (CE/protein association ratio) was positively correlated with the level of apoE expression in all cells for both LDL ($r^2 = 0.977$) and HDL_3 ($r^2 = 0.998$). We also show that, although in normal and low apoE expressor cells, 92% of LDL- and 80% HDL_3-CE hydrolysis is sensitive to chloroquine suggesting a pathway linked to lysosomes for both lipoproteins, cells overexpressing apoE lost 60% of chloroquine-sensitive HDL_3-CE hydrolysis without affecting that of LDL-CE. Thus, the level of apoE expression in HepG2 cells determines the fate of LDL and HDL_3.

Apolipoprotein (apo)[1] E, a 299 amino acid protein (*1*), is a constituent of chylomicrons, very low-density lipoproteins (VLDL), and their remnants. It is also found associated with two subclasses of high-density lipoproteins (HDL) known as HDL_1 and HDL_2 but not with HDL_3. At least 75% of plasma apoE is of hepatic origin (*2*), which is coherent with the role of this organ in lipoprotein synthesis. In HepG2 cell, a human hepatoma cell line considered as a good hepatic model (*3*), 75% of synthesized apoE was found to be secreted in the medium, 20% to be localized in the cell and 5% associated at the cell surface (*4*). Approximately 25% of this HepG2 cell-surface apoE is associated with the extracellular matrix, the remaining apoE being associated with the plasma membrane (5). In both cases, apoE is associated with proteoglycans.

ApoE is a ligand for the low-density lipoprotein (LDL)-receptor (LDLr) (*6*) and for the LDLr-related protein (LRP) (*7*), two receptors found on hepatic cells that mediate the endocytosis and complete degradation of lipoproteins (holoparticle uptake pathway). ApoE secreted by hepatic cells is known to play a role in the metabolism of lipoprotein containing apoE such as chylomicron remnants (reviewed in ref *8*). However, very few studies were aimed at elucidating the role of apoE expression on the metabolism of apoE-poor lipoproteins such as LDL and HDL_3, two classes of lipoproteins that follow both holoparticle (*9*, *10*) and CE-selective uptake (*11*, *12*) pathways. The latter pathway does not lead to the uptake and degradation of the entire lipoprotein, but only to the transfer of the lipoprotein-CE to the cell by a poorly understood mechanism. It is recognized that most of HDL-CE enters the cell by a selective uptake pathway linked to the scavenger receptor class B type I (SR-BI) identified in rodents (*13*) and to its human homologue known as CD36- and LIMPII-analogous-1 (CLA-

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[1] Abbreviations: Apo, apolipoprotein; B_{max}, maximal binding capacity; BSA, bovine serum albumin; CE, cholesteryl ester; CHO, chinese hamster ovary; CLA-1, CD36- and LIMPII-analogous-1; EDTA, ethylenediaminetetraacetate; FBS, fetal bovine serum; HDL_3, high-density lipoprotein-3; K_d, dissociation constant; LDL, low-density lipoprotein; LDLr, LDL receptor; LPDS, lipoprotein depleted serum; LRP, LDLr-related protein; MEM, minimal essential medium; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SR-BI, scavenger receptor class B type I; TBS, tris-buffered saline; TLC, thin-layer chromatography; VLDL, very low-density lipoprotein.

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1) (*14*). Degradation of HDL_3, by a holoparticle pathway, also occurs but the hepatic receptor involved is not clearly defined (reviewed in ref *15*). In the hepatic cell system, Ji et al. (*16*) reported that preincubating HDL with human apoE increases HDL holoparticle uptake, but not CE-selective uptake by rat hepatoma McA-RH7777 cells. In HepG2 cells, Fragoso and Skinner (*17*) have compared the ability of apoE-rich and apoE-poor subfractions of HDL to associate to HepG2 cells and came to the conclusion that apoE is not directly involved in the CE-selective uptake process, while using the monoclonal antibody anti-human apoE 1D7, Leblond and Marcel (*18*) came to the conclusion that cell-associated apoE is involved in HDL-CE selective uptake. Thus, the studies on HDL metabolism in regards to apoE lead to conflicting results. Furthermore, none of these studies on HDL addresses directly the effect of various levels of apoE expression by hepatic cells on both the HDL holoparticle and CE-selective uptake pathways and the fate of HDL-CE.

CE-selective uptake from LDL has also been shown in HepG2 cells (*11*), and it was shown that chinese hamster ovary (CHO) cells overexpressing SR-BI can selectively take CE from LDL (*19*). However, it is not yet known if under normal conditions SR-BI/CLA-1 is responsible for this activity in the hepatic cell. Indeed, HDL-CE was shown to be hydrolyzed in a nonlysosomal compartment (*20*), while CE coming by a selective uptake from LDL are metabolized in lysosomes (*11*). This is reviewed in more details in ref *21*. To date, there is no report on the role of hepatic cell-secreted apoE on LDL metabolism. The only available information was derived from other cell systems (*22*, *23*). Our aim was to define the role of HepG2 cell-secreted apoE on both LDL and HDL_3 metabolism by comparing HepG2 cells expressing various apoE levels to normal HepG2 cells. Thus, we created HepG2 cells expressing higher levels of apoE with an expression vector containing the full human apoE cDNA and HepG2 cells expressing lower levels of apoE by a constitutive antisense RNA technology. Up to 85% inactivation and 187% overexpression of apoE expression were achieved compared to normal HepG2 cells. The apoE level was found to be positively correlated with HDL_3 holoparticle uptake and LDL- and HDL_3-CE selective uptake. LDL holoparticle uptake was, however, higher in low expressor cells than in normal cells. Furthermore, our data reveal that overexpressing apoE in HepG2 cells reduces the chloroquine-sensitive hydrolysis of HDL_3-CE without modifying that of LDL-CE. Thus, in HepG2 cells, apoE expression level has a similar, yet not identical, effect on LDL and HDL_3 metabolism.

EXPERIMENTAL PROCEDURES

Materials. Human plasma was obtained from the Royal Victoria Hospital (Montréal, Québec, Canada). The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Rockville, MD). Purified human apoE came from Calbiochem (La Jolla, CA). Minimal essential medium (MEM), Geneticin (G418 sulfate), penicillin-streptomycin, and trypsin used for cell culture were from Life Technologies Gibco BRL (Burlington, Ontario, Canada). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT) while L-glutamine, fatty acid-free or regular bovine serum albumin (BSA) (fraction V), phenylmethanesulfonylfluoride (PMSF), anti-mouse IgG antibody, and ampicillin were obtained from Sigma Chemical Co (St-Louis, MO). [^{125}I] (as sodium iodide, 100 mCi/mL)- and [^{3}H]-cholesteryl oleate (30−60 Ci/mmol) were bought, respectively, from Amersham (Oakville, Ontario, Canada) and ICN Biomedical (Montreal, Quebec, Canada). Restriction enzymes and modification enzymes were obtained from Pharmacia (Montréal, Québec, Canada). pRc/CMV and pBluescript II KS+ plasmids came from Invitrogen (San Diego, CA). The vector pJS382 was a gift from Dr. Jonathan Smith (Rockefeller University, New York), while the vector pTV194 came from Dr. Robert Mahley (Gladstone Foundation, San Francisco).

Preparation of Stable Transformants Expressing Various Levels of ApoE. To obtain HepG2 cells deficient in apoE expression, pTV194 (*24*), a vector containing the human apoE3 cDNA, was digested with *Hin*dIII and *Eco*RI, resulting in the obtention of 1.2, 1.3, and 3 kbp fragments. The 1.3 kbp fragment, which contains the full-length apoE cDNA, was subcloned in the pBluescript KS+ vector and sequenced to confirm its identity. Thereafter, the fragment was excised with *Xba*I and *Hin*dIII while, in parallel, the pRc/CMV expression vector was digested with the same enzymes. Ligation between the fragment and the vector was accomplished at 20 °C with T4 DNA ligase followed by transformation in *Escherichia coli* DH5α. A fraction of the recombinant DNA was digested by *Not*I and *Hin*dIII in order to excise a fragment of 831 bp at the 3′ end of the apoE cDNA and the plasmid was ligated. Thus, two vectors were created, one containing the complete apoE cDNA and another containing the first 474 bp of the cDNA, both inserted in the antisense orientation. To create cells overexpressing apoE, pJS382, a vector containing the full human apoE3 cDNA, was digested with *Xba*I and 1.2 and 2.4 kbp fragments were obtained. The 1.2 kbp fragment was inserted in the pRc/CMV expression vector opened with *Xba*I. The three different constructs and the expression vector without insert were separately transfected in HepG2 cells using the standard calcium phosphate method. G418-resistant cell lines were selected and isolated from the pools with cloning cylinders and then propagated to study the apoE expression level. All stable transformants resemble the native HepG2 cells in appearance and in growth rate.

Cell Culturing. HepG2 cells were grown in 75-cm^2 flasks containing 15 mL of MEM supplemented with 10% (v/v) FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (4 mM), as previously described (*11*). Medium was changed every 2 days, and cells were propagated every 7 days. Three days prior to the binding, association, and degradation studies, 4.5×10^5 cells were seeded in 3.8 cm^2 culture dishes (12 well-dishes) for binding, association and degradation assays. The cells were approximately 90% confluent.

ApoE, LDLr, and SR-BI/CLA-1 Protein Estimation by Immunoblotting. Cell medium was changed and harvested 66 h later to measure apoE secretion. Also, cell proteins were obtained by the method of Yoshimura et al. (*25*). Proteins (200−400 μg) were separated by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*26*) followed by Western blotting to nitrocellulose paper according to the method of Burnette (*27*). The nitrocellulose

paper was incubated in Tris-buffered saline (TBS) containing the monoclonal anti-human apoE antibody 6C5 (1:500) (*28*) for 90 min at 37 °C. The nitrocellulose was washed with TBS and then incubated with ^{125}I-labeled anti-mouse IgG (10 μg/mL, 1×10^6 cpm) for 90 min at 37 °C. Thereafter, the nitrocellulose paper was extensively washed with TBS and an autoradiogram was obtained by exposing the dried nitrocellulose paper to Kodak XAR film at −80 °C. The intensity of the autoradiogram signal was quantified by densitometry using the Imagequant system of Molecular Dynamics (Sunnyvale, CA). Immunoblotting experiments were also conducted with the cultured medium of control HepG2 cells and 0.5, 1.0, and 2.5 μg of purified human apoE in order to determine the rate of apoE synthesis by control HepG2 cells. LDLr and SR-BI/CLA-1 levels were determined from cellular proteins with a rabbit polyclonal anti-human LDLr antibody (Research Diagnostics) for LDLr receptor estimation and a rabbit polyclonal anti-murine SR-BI antibody (Novus Biologicals) for SR-BI/CLA-1, respectively.

Preparation and Radiolabeling of Lipoproteins. To the plasma was added 0.01% (w/v) of ethylenediamine tetraacetate (EDTA), 0.02% (w/v) of sodium azide and 10 μM of PMSF before the isolation of lipoproteins, which was achieved by ultracentrifugation as described by Hatch and Lees (*29*). Human LDL (density 1.025−1.063 g/mL) and HDL_3 (density 1.125−1.21 g/mL) were prepared as described by Brissette and Noël (*30*). Both lipoproteins contained no detectable amounts of apoE, as assessed by SDS−PAGE. LDL and HDL_3 were iodinated by a modification (*31*) of the iodine monochloride method of McFarlane (*32*). One millicurie of sodium 125-iodide was used to iodinate 2.5 mg of LDL or HDL_3 in the presence of 30 or 10 nmol, respectively of iodine monochloride in 0.5 M glycine-NaOH, pH 10. Free iodine was removed by gel filtration on Sephadex G-25 followed by an overnight dialysis in TBS. The specific radioactivity ranged 115000−200000 cpm/μg of protein. LDL and HDL_3 were radiolabeled in their CE with [^{3}H]cholesteryl oleate as described by Roberts et al. (*33*). Briefly, 25 μCi of [^{3}H]CE was evaporated under nitrogen and resuspended in 250 μL of acetone. This volume of acetone was added dropwise to 3 mL of lipoprotein depleted serum (LPDS) in a glass tube at room temperature and acetone was evaporated under a gentle stream of nitrogen. One milliliter of lipoprotein at a concentration of 1 mg protein/mL was added to the LPDS containing [^{3}H]-CE and the mixture was incubated for 30 min at 4 °C. Thereafter, the labeled lipoproteins were reisolated by ultracentrifugation. The specific activity of lipoproteins labeled in CE ranged from 7500 to 20 000 dpm/μg protein.

Binding Assay. The cells were washed twice with 1 mL of phosphate-buffered saline (PBS) and were then incubated for 2 h at 4 °C with 0−50 μg of protein/mL of [^{125}I]LDL or HDL_3 in 125 μL of MEM containing 4% (w/v) fatty-acid free BSA and 50 mM Hepes, pH 7.4, in a total volume of 250 μL (total binding). Nonspecific binding was determined by the addition of 1 mg of protein/mL of the proper unlabeled lipoprotein. At the end of the incubation, the cell monolayers were washed two times with 1 mL of PBS containing 0.2% (w/v) BSA (PBS−BSA), then twice with 1 mL of PBS. The cells were solubilized in 1.5 mL of 0.1 N NaOH, assayed for protein content and counted for radioactivity in a Cobra II counter (Canberra-Packard). The specific binding was calculated by subtracting the nonspecific binding of ^{125}I-labeled lipoproteins from the total binding. The curves generated by the specific-binding data were transformed into plots of the ratio of cell-bound to free ^{125}I-labeled lipoproteins versus cell-bound ^{125}I-labeled lipoproteins, according to the method of Scatchard (*34*). The dissociation constant (K_d) was calculated from the slopes and the maximum binding capacity (B_{max}) was obtained from the *x*-axis intercept.

Cell Association and Degradation Assays. HepG2 cell association of [^{125}I]lipoprotein and [^{3}H]CE-lipoprotein (15 μg of protein/mL) lasted for 3 h at 37 °C, as for the binding studies but without Hepes. At the end of the incubation, dishes were processed as for the binding studies. Association data were obtained from an estimate of the radioactivity of the washed cells resuspended in 0.1 N NaOH. The results are expressed in [^{125}I]lipoprotein−protein/mg of HepG2 cell protein. Associated [^{3}H]CE was quantitated with a beta-counter (Wallach-Fisher). To compare the association of lipoproteins labeled in protein (^{125}I) or in CE (^{3}H), the association data of [^{3}H]CE-lipoprotein were estimated as protein associated/mg cellular protein. To achieve this, the specific activity of [^{3}H]CE-LDL or [^{3}H]CE-HDL_3 was established in disintegrations per minute per microgram of LDL or HDL_3 protein. Selective uptake is demonstrated when the ratio of [^{3}H]CE-lipoprotein (association)/[^{125}I]lipoprotein (association + degradation) is greater than unity. The degradation of the proteins of [^{125}I]lipoprotein was estimated from the medium. Essentially, trichloroacetic acid (TCA) was used at a final concentration of 12% and degradation was estimated as the TCA-soluble fraction. Proper controls without cells were done to account for free ^{125}iodine. Some experiments were conducted after a preincubation for 2 h with 1.5 units/mL of chondroitinase ABC and 4.5 units/mL of heparinase I.

CE Hydrolysis Assays. Cells were washed twice with 1 mL of PBS and were then preincubated for 1 h at 37 °C in the medium used in the association assay but in the presence or absence of 100 μM of chloroquine. After the preincubation period, 40 μg of protein/mL of [^{3}H]CE-LDL or HDL_3 were added to the wells and cells were incubated for 4 h. Nonspecific CE-hydrolysis was determined by the addition of 1 mg of protein/mL of the proper unlabeled lipoprotein. At the end of the incubation, the cell monolayers were washed as for the binding studies. The lipids were extracted in situ following two 30 min incubations with 1 mL of hexane/isopropylic alcohol (3:2, v/v). Cells were solubilized in 1.5 mL of 0.1 N NaOH and assayed for protein content. The lipid extracts were dried under N_2, resuspended in 50 μL of chloroform and separated by thin-layer chromatography on silica gel G plates run in petroleum ether/diethyl ether/acetic acid (90:10:1, v/v). The plates were dried and the lipid spots were revealed by exposure to iodine vapor. Following complete disappearance of I_2, spots corresponding to the free cholesterol and CE positions were scraped from the plates and quantified by liquid-scintillation counting.

Other Methods. Protein content was determined by the method of Lowry et al. (*35*) with BSA as standard. Two-tailed Student's unpaired and paired *t*-test were used to determine significant differences between assays.

RESULTS

Our primary goal was to create HepG2 cells expressing higher and lower levels of apoE compared to normal HepG2

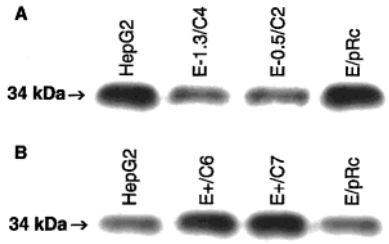

FIGURE 1: Immunoblot analysis of apoE levels in different subtypes of HepG2 cells.Cells were solubilized with 1% Triton X-100 and 400 μg (A) or 200 μg (B) of proteins were separated by 10% SDS−PAGE and transferred onto nitrocellulose membrane. The level of apoE was estimated by immunodetection with 6C5, a monoclonal anti-human apoE antibody, as described in the Experimental Procedures.

cells. We determined by quantitative immunoblotting that normal HepG2 cells secrete 0.25 μg/mg of cell protein/h. To obtain cells expressing high levels of apoE, we constructed an expression vector containing the full-length apoE cDNA in the sense orientation. Low expressor cells were created with a vector producing apoE antisense RNA. Since we could not determine empirically the length of the antisense RNA that would be the most efficient, we used the full-length apoE cDNA or the first 474 bp of the same cDNA. Both were inserted in the antisense orientation in pRc/CMV. Stable transformants were named according to the orientation of their cDNA insert and, in the case of low apoE expressing cells, the length of their cDNA insert. Thus, cells that express the human apoE cDNA were named E+, while cells expressing antisense RNA were named E−1.3 if they received the full length cDNA, or E−0.5 if they received the first 474 base pairs of the cDNA. HepG2 cells were also transfected with the vector pRc/CMV without insert. These cells were named E/pRc. Cellular clones were obtained from each pool of transformants, and their level of apoE was determined by immunoblotting. Figure 1 shows the level of apoE expression in normal HepG2 cells and E/pRc and in our two best overexpressing clones (E+/C6 and E+/C7) and two most deficient clones (E−1.3/C4 and E−0.5/C2). While the level of apoE in E/pRc is not different from the normal HepG2 cells, densitometric analysis of four different experiments taking the HepG2 cell apoE level as 100% revealed that E−1.3/C4 and E−0.5/C2 cells express 15% ± 3 (mean ± SD) and 24% ± 4 of the level of normal HepG2 cells, respectively, while E+/C6 and E+/C7 cells have apoE levels corresponding to 226% ± 6 and 287% ± 8 of the control level, respectively. Thus, there is a 19-fold difference in apoE expression between our most deficient and best overexpressing cells. The measurement in the medium followed the same type of modulation in the level of apoE (data not shown). Expression levels of LDLr and SR-BI/CLA-1 were determined by immunoblotting in E−1.3/C4, E+/C7, and control HepG2 cells. While the LDLr level was the same, a 30% reduction in SR-BI/CLA-1 level was detected in E−1.3/C4 compared to the two other cell types.

Our next goal was to define if the changes of apoE levels in HepG2 cells would affect the ability of HepG2 cells to bind LDL and HDL_3, two lipoprotein classes that can be isolated without detectable amounts of apoE (*30*). Figure 2A shows that the specific binding of [^{125}I]LDL is more important for the cells expressing low levels of apoE than in other cells. The Scatchard plots (panel B) show that the *x*-intercept is greater in the case of low expressing cells compared to the other cells while the slopes are the same no matter the level of apoE expression by the cells. The binding parameters calculated for each experiment are shown in Table 1. This table reveals that the maximal binding capacity (B_{max}) of [^{125}I]LDL is statistically increased ($p < 0.01$) by 126% and 78% for E−1.3/C4 and E−0.5/C2 cells, respectively, in comparison with the B_{max} of control HepG2 cells. No differences were found between normal and apoE overexpressing cells. Analysis of the dissociation constants (K_d) indicates that a variation in the apoE level has no effect on the affinity of the LDL binding to HepG2 cells. Similar results were obtained for the binding of [^{125}I]HDL_3 to the different subtypes of HepG2 cells as seen in Figure 2, panels C and D. Table 1 shows that the B_{max} of [^{125}I]HDL_3 is statistically increased ($p < 0.001$) by 83 and 53% for E−1.3/C4 and E−0.5/C2 cells, respectively, compared to that of normal cells. In contrast with the observation made with LDL, low apoE expressing cells have a significantly reduced ($p < 0.01$) affinity for HDL_3 as seen by the 39% and 30% increases in the K_d with E−1.3/C4 and E−0.5/C2 cells, respectively. Thus, apoE secreted by HepG2 cells transforms HDL_3, but not LDL, into a higher affinity ligand for cell receptors, while at lower level than normal, apoE favors LDL and HDL_3 binding to HepG2 cells.

Thereafter, we analyzed the metabolism of LDL by these cells, in function of apoE expression. The experiments were conducted at 37 °C, a temperature that allows internalization and degradation of lipoproteins after their binding to cell membranes. Table 2 shows that E−1.3/C4 and E−0.5/C2 cells degrade 51% ($p < 0.05$) and 108% ($p < 0.001$) more [^{125}I]LDL-protein, respectively, than normal HepG2 cells. A parallel effect was observed for LDL-protein association. These results are in agreement with those obtained with the binding studies (Table 1). Association assays were conducted after a preincubation in the absence or presence of heparinase I and chondroitinase ABC in order to evaluate the role of proteoglycans on the cell surface. These enzymes had no effect on [^{125}I]LDL association to our low apoE expressor cells as 101.6 ± 2.7% (mean ± SD, $n = 5$) of the association level of cells preincubated without the enzymes was measured. However, the enzymatic treatment on high apoE expressor cells increased LDL association to 130.4 ± 10.1% ($n = 6$). This statistically significant increase ($p < 0.05$) suggests that apoE on proteoglycans of high expressor cells reduces LDL ability to associate with HepG2 cells.

The association of the lipid moiety of LDL, more precisely CE, was also investigated. For this, LDL were labeled with [^{3}H]CE. Table 3 shows that [^{3}H]CE-LDL cell-association was significantly increased ($p < 0.001$) by 87 and 129% in E+/C6 and E+/C7 cells, respectively, while the association of LDL-CE with cells expressing low levels of apoE was not statistically different from that of normal HepG2 cells. Analysis of the CE/protein association ratio, an indicator of the LDL-CE selective uptake, reveals that the level of apoE expression in HepG2 cells is highly correlated ($r^2 = 0.977$) with this pathway. Indeed, compared to the lowest expressing cells, our highest expressing cells are 3-fold better to selectively take up CE from LDL. Thus, the information gathered on LDL metabolism reveals that subnormal levels of HepG2-apoE favors the holoparticle pathway (LDL-

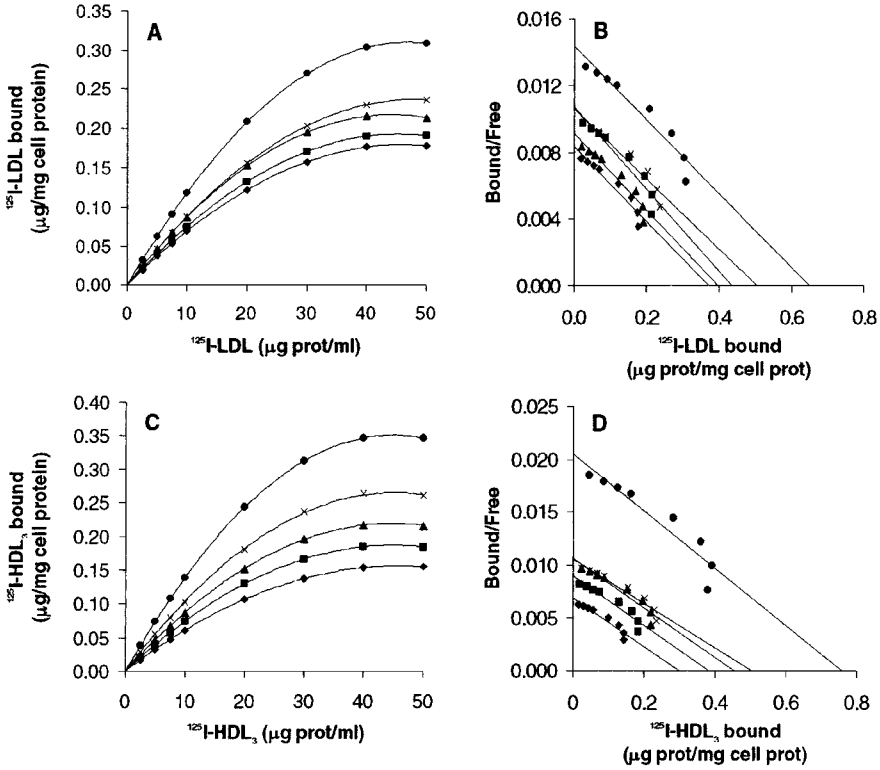

FIGURE 2: Specific binding of [^{125}I]LDL and [^{125}I]HDL$_3$ to different subtypes of HepG2 cells. Saturation curves of the specific binding of [^{125}I]LDL (A) or [^{125}I]HDL$_3$ (C) to either normal HepG2 (◆), E−1.3/C4 (×), E−0.5/C2 (●), E+/C6 (■) or E+/C7 (▲) cells and Scatchard plots of the specific binding of [^{125}I]LDL (B) or [^{125}I]HDL$_3$ (D). To measure the total binding on these cells, [^{125}I]lipoproteins (0−50 μg of protein/mL) were incubated at 4 °C for 2 h with HepG2 cells. The nonspecific binding was measured by adding 1 mg of protein/mL of the proper unlabeled lipoprotein and the specific binding was calculated by subtracting the nonspecific binding from the total binding. These are representative experiments conducted in duplicate.

Table 1: Parameters of LDL and HDL$_3$ Binding to the Different HepG2 Cell Subtypes[a]

lipoprotein	cell	apoE expression level (%)	K_d (μg of protein/mL)	B_{max} (ng of protein/mg of cell protein)
[^{125}I]LDL	E−1.3/C4	15	42.0 ± 3.1	713.0 ± 102.8[b]
	E−0.5/C2	24	45.1 ± 3.7	560.4 ± 71.8[b]
	HepG2	100	43.4 ± 3.5	315.6 ± 22.4
	E+/C6	226	42.3 ± 3.1	380.1 ± 33.4
	E+/C7	287	42.3 ± 1.2	396.1 ± 40.8
[^{125}I]HDL$_3$	E−1.3/C4	15	69.6 ± 6.6[b]	714.9 ± 52.2[c]
	E−0.5/C2	24	65.3 ± 8.0[b]	596.2 ± 43.6[c]
	HepG2	100	50.1 ± 4.1	390.5 ± 20.9
	E+/C6	226	51.2 ± 8.5	402.1 ± 41.2
	E+/C7	287	50.7 ± 9.7	421.9 ± 44.9

[a] Binding assays of ^{125}I-labeled lipoproteins to the different subtypes of HepG2 cells were carried out as described in the Experimental Procedures. Briefly, [^{125}I]lipoproteins (0−50 μg of protein/mL) were incubated with cells at 4 °C for 2 h. Nonspecific binding was measured by adding 1 mg of protein/mL of the proper unlabeled lipoprotein and specific binding was calculated by subtracting the nonspecific from the total binding. K_d and B_{max} were determined from Scatchard plots of the specific binding data. Each value represents the mean ± SD of five experiments conducted each in duplicate. Statistical differences were determined with a paired *t*-test. [b] Statistically different ($p < 0.01$) from the results obtained with normal HepG2 cells. [c] Statistically different ($p < 0.001$) from the results obtained with the normal HepG2 cells.

protein binding, association and degradation) while higher levels optimize the LDL-CE selective uptake pathway.

Similar experiments were conducted with HDL$_3$. Confirming our previous data (*39*), normal HepG2 cells can degrade (Table 3) and associate HDL$_3$-protein and also selectively take CE from HDL$_3$, but differently from LDL. Indeed, HDL$_3$-protein degradation (Table 2), -protein, and -CE association (Table 3) are 6, 54, and 39% that of LDL, respectively. Table 2 shows that, differently from LDL degradation, HDL$_3$-protein degradation is positively correlated ($r^2 = 0.989$) with the expression of apoE in HepG2 cells. Indeed, the E−1.3/C4 and E−0.5/C2 cells degrade 36% ($p < 0.01$) and 29% ($p < 0.001$) less HDL$_3$-protein, respectively, than normal HepG2 cells while the E+/C6 and E+/C7 cells degrade 49% ($p < 0.01$) and 59% ($p < 0.05$) more HDL$_3$-protein, respectively, than normal HepG2 cells. In agreement with our findings with LDL, there is a significant increase ($p < 0.001$) of 185 and 228% of [^{125}I]-HDL$_3$ cell-association with E−1.3/C4 and E−0.5/C2 cells, respectively, while the association of HDL$_3$ to cells overexpressing apoE was not significantly different from the control cells (Table 2). Again, these results are in agreement with those obtained with the binding studies (Table 1). Table 3 shows a significantly higher [^{3}H]CE-HDL$_3$ association ($p < 0.05$) of 58 and 70% for E+/C6 and E+/C7 cells, respectively, while the same association is measured in low apoE expressing and control cells. As for LDL, CE-selective uptake is highly correlated ($r^2 = 0.998$) with the level of apoE, revealing that the apoE level is also important for HDL$_3$-CE selective uptake by HepG2 cells. As a matter of fact, CE-selective uptake values in E- cells do not differ from unity. Thus, in those cells a reduction of 76% in apoE expression virtually abolished HDL$_3$-CE selective uptake.

The intracellular fate of lipoprotein-CE in conjunction with the apoE expression levels was investigated. For this purpose,

Table 2: Association and Degradation of [^{125}I]Lipoprotein by Different HepG2 Cell Subtypes[a]

lipoprotein	cell	apoE expression level (%)	[^{125}I]lipoprotein degradation (ng of protein/ mg of cell protein)	[^{125}I]lipoprotein association (ng of protein/ mg of cell protein)	degradation/ association ratio
^{125}I-LDL	E−1.3/C4	15	30.0 ± 3.2[c]	94.3 ± 24.2[c]	0.38 ± 0.10
	E−0.5/C2	24	21.8 ± 5.4[c]	60.4 ± 20.6[d]	0.44 ± 0.13
	HepG2	100	14.4 ± 12.6	37.6 ± 14.1	0.42 ± 0.14
	E+/C6	226	13.2 ± 4.0	50.8 ± 20.9	0.29 ± 0.11[d]
	E+/C7	287	13.4 ± 5.7	64.0 ± 29.7	0.23 ± 0.07[d]
^{125}I−HDL$_3$	E−1.3/C4	15	0.52 ± 0.17[d]	69.9 ± 36.9[b]	0.010 ± 0.00[d]
	E−0.5/C2	24	0.58 ± 0.29[d]	60.7 ± 31.0[b]	0.012 ± 0.00[d]
	HepG2	100	0.81 ± 0.26	21.3 ± 6.5	0.046 ± 0.028
	E+/C6	226	1.21 ± 0.24[d]	24.7 ± 7.8	0.062 ± 0.032[c]
	E+/C7	287	1.29 ± 0.40[c]	22.8 ± 7.0	0.061 ± 0.036[c]

[a] Association and degradation assays were conducted as described in the Experimental Procedures with 15 μg of protein/mL of either [^{125}I]LDL or [^{125}I]HDL$_3$. Degradation of LDL- or HDL-protein was measured in the medium. Incubation was conducted in parallel with 1 mg of protein/mL of the proper unlabeled lipoprotein to determine the level of nonspecific association or degradation. Each value represents the mean ± SD of 10 experiments conducted each in triplicate. Statistical differences were determined with a paired *t*-test. [b] Statistically different ($p < 0.05$) from the results obtained with normal HepG2 cells. [c] Statistically different ($p < 0.01$) from the results obtained with normal HepG2 cells. [c] Statistically different ($p < 0.001$) from the results obtained with normal HepG2 cells.

Table 3: Protein and Cholesteryl Ester Association of LDL and HDL$_3$ with Different HepG2 Cell Subtypes[a]

lipoprotein	cell	apoE expression level (%)	[^{125}I]lipoprotein association (ng of protein/ mg of cell protein)	[^{3}H]CE association (ng of protein/ mg of cell protein)	CE-selective uptake
[^{125}I]LDL	E−1.3/C4	15	100.1 ± 25.6[b]	144.5 ± 73.1	1.68 ± 0.42[b]
	E−0.5/C2	24	69.9 ± 22.5[b]	153.5 ± 65.2	2.21 ± 0.53[c]
	HepG2	100	39.6 ± 12.6	123.6 ± 55.1	3.17 ± 1.11
	E+/C6	226	46.2 ± 18.9	231.1 ± 58.8[b]	4.86 ± 1.04[d]
	E+/C7	287	60.5 ± 24.0	282.7 ± 90.7[b]	5.14 ± 0.68[d]
[^{125}I]HDL$_3$	E−1.3/C4	15	69.9 ± 36.9[c]	42.6 ± 16.9	0.73 ± 0.19[b]
	E−0.5/C2	24	60.7 ± 30.9[c]	42.5 ± 20.0	0.84 ± 0.29[b]
	HepG2	100	21.3 ± 6.5	47.8 ± 14.3	2.37 ± 0.50
	E+/C6	226	24.7 ± 7.8	75.6 ± 22.7[d]	3.47 ± 0.59[c]
	E+/C7	287	22.8 ± 7.0	81.2 ± 25.4[d]	4.02 ± 0.93[c]

[a] Cells were incubated for 3 h at 37 °C with either 15 μg/mL of [^{125}I]lipoprotein or [^{3}H]CE-lipoprotein and cell association was measured as described in the Experimental Procedures. Incubation was conducted in parallel with 1 mg of protein/mL of the proper unlabeled lipoprotein to determine the level of nonspecific association. Each value represents the mean ± SD of 10 experiments conducted each in triplicate. Statistical differences were determined with a paired *t*-test. [b] Statistically different ($p < 0.001$) from the results obtained with normal HepG2 cells. [c] Statistically different ($p < 0.05$) from the results obtained with normal HepG2 cells. [d] Statistically different ($p < 0.01$) from the results obtained with normal HepG2 cells.

hydrolysis of LDL- and HDL$_3$-CE was analyzed in the presence of chloroquine, an inhibitor of the lysosomal function, in control cells and in our highest and lowest apoE expressing cells. Figure 3 shows that LDL-CE or HDL$_3$-CE hydrolysis is not statistically different between E−1.3/C4, E+/C7, and normal HepG2 cells in the absence of chloroquine. Panel A reveals that chloroquine has a similar effect on the three cell lines, significantly reducing LDL-CE hydrolysis by an average of 92% ($p < 0.001$). Important differences were detectable between sensitivity of LDL- and HDL$_3$-CE hydrolysis in the presence of chloroquine as panel B shows that chloroquine significantly reduces ($p < 0.001$) HDL$_3$-CE hydrolysis by 72 and 86% in E−1.3/C4 and normal HepG2 cells, respectively, while it only decreases HDL$_3$-CE hydrolysis by 35% in cells overexpressing apoE. Thus, an apoE level higher than normal reduces the sensitivity of HDL$_3$-CE hydrolysis to chloroquine without changing that of LDL.

DISCUSSION

Our goal was to define the role that hepatic cell-apoE plays in LDL and HDL$_3$ metabolism. A hepatic-derived cell was chosen since most of the lipoprotein metabolism occurs in the liver. To strengthen our study, we chose to compare HepG2 cells expressing normal, low and high levels of apoE. Thus, we constructed apoE expression vectors in order to obtain HepG2 cells overexpressing apoE and we also created HepG2 cells that constitutively express apoE antisense RNA to reduce apoE expression in HepG2 cells.

The study of LDL binding to the different HepG2 cell subtypes revealed that reducing the apoE level below the control level increases the binding capacity (B_{max}) of LDL, while increasing apoE level has no effect. A parallel effect was demonstrated on LDL-protein association and degradation. This cannot be attributed to higher levels of LDLr in apoE deficient cells since the LDLr expression was the same in all types of cells. Variations of the apoE levels did not modify the affinity (K_d) of LDL for these cell lines. Thus, it has to be concluded that HepG2 cells expressing lower levels of apoE than the control level metabolize more LDL by the endocytosis/degradation pathway. These results were unexpected since there were many reasons to believe that higher levels of apoE would favor the binding/association/degradation pathway by the LDLr following the acquisition of

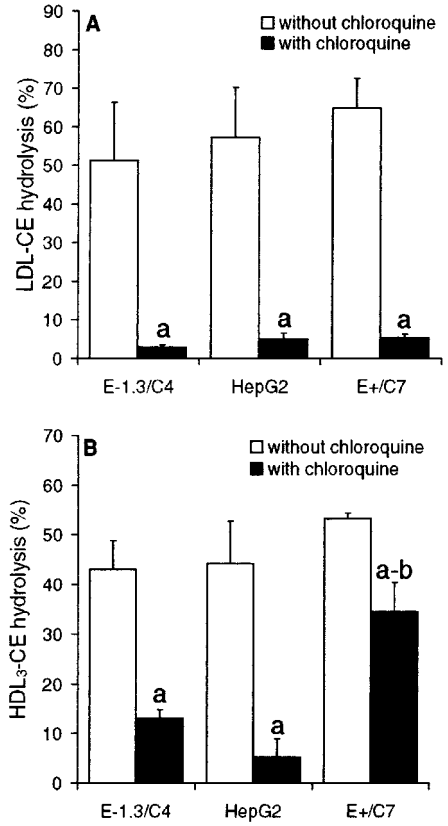

FIGURE 3: Hydrolysis of [H^3]CE-LDL and [H^3]CE-HDL_3 in different subtypes of HepG2 cells. Cells were preincubated for 1 h at 37 °C in the absence or presence of 100 μM of chloroquine. After the preincubation period, 40 μg of protein/mL of [^{3}H]CE-LDL or [^{3}H]CE-HDL_3 were added to the wells and CE hydrolysis was measured as described in the Experimental Procedures. Incubation was conducted in parallel with 1 mg of protein/mL of the proper unlabeled lipoprotein to determine the level of nonspecific CE hydrolysis. Each value represents the mean ± SD of four experiments conducted each in duplicate. Statistical differences were determined with unpaired *t*-test. (a) Statistically different ($p < 0.01$) from the results obtained with the same cell line without chloroquine. (b) Statistically different ($p < 0.01$) from the results obtained with normal HepG2 cells.

hepatic apoE by LDL, as suggested by the study of Choi et al. (*22*) with CHO cells overexpressing apoE. The difference can depend either on the different cells used (type or species) or on the levels of apoE expression. Knowing that apoE is found associated with proteoglycans (*5*), we have investigated the role of such molecules on low and high apoE expressor cells. Our results show that heparinase I and chondroitinase ABC do not modify LDL association to HepG2 cells expressing low levels of apoE. Therefore, it appears that proteoglycans do not play a significant role in LDL metabolism in that cell subtype. Interestingly, the same treatment on high apoE expressor cells increases LDL association, suggesting that proteoglycans bearing apoE reduces LDL association to the cell. Other experiments are required to define the mechanism involved.

Study of HDL_3 binding/association showed a similar pattern than that observed for LDL suggesting that our conclusion on proteoglycans free of apoE applies also to HDL_3. Our degradation experiment is in agreement with the results of Ji et al. (*16*), as a very strong positive correlation was found between HDL_3-protein degradation and the various apoE levels of our cells. It can be speculated that the effect observed on HDL_3-protein degradation relates to an enrichment of HDL_3 in apoE from the medium which would then redirect HDL_3 to an apoE receptor such as the LDLr or the LRP for rapid endocytosis and degradation. This is supported by the demonstration of Garcia et al. (*10*) that HDL_3 are internalized in HepG2 cells by a classical endocytic pathway. Alternatively, the HDL-receptor responsible for the HDL holoparticle uptake that remains to be clearly identified in the liver (*15*), could see its degradation activity increased with apoE.

Other experiments were conducted to determine the role of hepatic apoE in the selective uptake and processing of CE from LDL and HDL_3. For both lipoproteins, the pattern of CE association was the inverse of protein association. In other words, instead of increasing in apoE-deficient cells compared to normal cells, CE association rose in cells overexpressing apoE. Altogether, these effects lead to an almost perfect correlation between apoE expression in HepG2 cells and CE-selective uptake from both lipoproteins. Our immunoblotting experiments reveal that this effect cannot be explained by different levels of SR-BI/CLA-1 in our different cell types. Our findings with LDL are similar to the findings of Swarnakar et al. (*23*) who showed that overexpression of human apoE in murine Y1 adrenocortical cells favors the CE-selective uptake, while the effect that we have detected with HDL_3 is in agreement with the results of Leblond and Marcel (*18*). From our results, it can be calculated that in our lowest and highest apoE expressing cells, 60 and 19% of LDL-CE enter the cell by the holoparticle pathway, respectively, the remaining entering by the CE-selective uptake pathway. Results are 100 and 25% for HDL_3-CE. In other words, it means that in low apoE expression cells the holoparticle pathway is favored while in high apoE expressor cells the vast majority of lipoprotein-CE is taken by a CE-selective uptake pathway. Our findings are supported by the knowledge that CE-selective uptake is important in cells that are good apoE expressor, such as adrenals and ovary cells. Thus, apoE can be considered as an activator of the CE-selective uptake pathway. Other experiments are needed to define the mechanism by which apoE exerts its effect. Another important issue is that apoE overexpression appears to target HDL_3-CE, but not LDL-CE, to a nonlysosomal compartment for hydrolysis as seen by the loss of chloroquine sensitivity. This suggests that even though high levels of apoE favor the CE-selective uptake pathway for both LDL and HDL_3, more than one receptor may be involved. It can therefore be proposed that, in normal HepG2 cells, LDL- and HDL_3-CE are entering the cells by the same selective uptake pathway that is highly chloroquine sensitive and likely linked to lysosomes, while in high apoE expression cells, HDL_3-CE but not LDL-CE are directed to SR-BI/CLA-1 known to lead to extralysosomal hydrolysis. If this is true then clarifying the identity of the receptor different from SR-BI/CLA-1 becomes a priority.

In conclusion, we were successful in reducing and increasing expression of apoE in HepG2 cells. We showed that lower levels of apoE increases binding and protein association of both LDL and HDL_3 compared to normal cells, while higher apoE levels increases CE association. ApoE level was found to be positively correlated with HDL_3, but not LDL, degradation. Both LDL- and HDL_3-CE selective uptake were positively correlated with the level of apoE expressed by

HepG2 cells. However, the selective uptake of CE from LDL and HDL_3, in cell overexpressing apoE, appears to be mediated by distinct receptors as in high apoE expressing cells, the hydrolysis of HDL_3-derived CE is largely chloroquine-insensitive whereas that of LDL-derived CE is predominantly chloroquine-sensitive. Therefore apoE has both similar and different effects on LDL and HDL_3 metabolism.

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