Cellular free cholesterol in Hep G2 cells is only partially available for down-regulation of low-density-lipoprotein receptor activity

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We have previously shown that in Hep G2 cells and human hepatocytes, as compared with fibroblasts, the low-density lipoprotein (LDL) receptor activity is only weakly down-regulated after incubation of the cells with LDL, whereas incubation with high-density lipoproteins (HDL) of density 1.16–1.20 g/ml (heavy HDL) strongly increased the LDL-receptor activity. To elucidate this difference between hepatocytes and fibroblasts, we studied the cellular cholesterol homeostasis in relation to the LDL-receptor activity in Hep G2 cells. (1) Interrupting the cholesteryl ester cycle by inhibiting acyl-CoA:cholesterol acyltransferase (ACAT) activity with compound 58-035 (Sandoz) resulted in an enhanced LDL-mediated down-regulation of the receptor activity. (2) The stimulation of the receptor activity by incubation of the cells with cholesterol acceptors such as heavy HDL was not affected by ACAT inhibition. (3) Incubation of the Hep G2 cells with LDL, heavy HDL or a combination of both grossly affected LDL-receptor activity, but did not significantly change the intracellular content of free cholesterol, suggesting that in Hep G2 cells the regulatory free cholesterol pool is small as compared with the total free cholesterol mass. (4) We used changes in ACAT activity as a sensitive (indirect) measure for changes in the regulatory free cholesterol pool. (5) Incubation of the cells with compactin (2 μ M) without lipoproteins resulted in a 4-fold decrease in ACAT activity, indicating that endogenously synthesized cholesterol is directed to the ACAT-substrate pool. (6) Incubation of the cells with LDL or a combination of LDL and heavy HDL stimulated ACAT activity 3–5-fold, whereas incubation with heavy HDL alone decreased ACAT activity more than 20-fold. Our results suggest that in Hep G2 cells exogenously delivered (LDL-) cholesterol and endogenously synthesized cholesterol are primarily directed to the cholesteryl ester (ACAT-substrate) pool or, if present, to extracellular cholesterol acceptors (heavy HDL) rather than to the free cholesterol pool involved in LDL-receptor regulation.

INTRODUCTION

The liver plays an important role in the removal of low-density lipoprotein (LDL) from the circulation (Steinberg, 1983; Brown & Goldstein, 1983). In normal subjects the removal of more than 70 % of LDL takes place via the LDL receptor. The presence of LDL receptors on human hepatocytes has been described by several groups (Harders-Spengel *et al.*, 1982; Kosykh *et al.*, 1985; Edge *et al.*, 1986). The importance of hepatic LDL receptors in controlling the plasma concentration of LDL cholesterol in humans has been clearly demonstrated by Bilheimer *et al.* (1984) after liver transplantation in an LDL-receptor-deficient homozygous familial-hypercholesterolaemic patient.

Since experimentation with primary cultures of human hepatocytes faces a major problem, that is the frequency with which pieces of fresh human liver can be obtained, we performed our present study with Hep G2 cells. This cell line has been shown, by several investigators, to express many specific liver-cell functions. We (Havekes *et al.*, 1983) and others (Wu *et al.*, 1984; Leichtner *et al.*, 1984; Dashti *et al.*, 1984) reported that Hep G2 cells also display LDL-receptor activity.

In extrahepatic cells the LDL-receptor activity and cellular cholesterol synthesis are much decreased after incubation of the cells with LDL (Goldstein & Brown,

1977). Since *in vivo* hepatocytes are exposed to physiological concentrations of LDL (Wisse, 1977) and the LDL receptor is active in the liver *in vivo* (Bilheimer *et al.*, 1984), we studied the regulation of the LDL receptor in liver cells in more detail (Havekes *et al.*, 1986a,b). We found that, in contrast with extrahepatic cells, in Hep G2 cells and in human hepatocytes the LDL-receptor activity is only weakly down-regulated by preincubation of the cells with fairly high concentrations of LDL, whereas in these cells the LDL-receptor activity is strongly stimulated by HDL of density > 1.16 g/ml (heavy HDL). These results suggest that, as far as the regulation of the LDL receptor is concerned, hepatocytes are more sensitive than fibroblasts with respect to heavy-HDL-mediated depletion of cholesterol from the hypothetical regulatory cholesterol pool, whereas hepatocytes seemed to be less effective in LDL-mediated delivery of extracellular cholesterol into the regulatory pool. Since in hepatocytes cholesterol is at least partly diverted into a cholesteryl ester pool on incubation with LDL (Suckling & Stange, 1985), and hepatocytes are known to use cholesterol for the synthesis of lipoproteins and bile acids (Thrift *et al.*, 1986; Ellsworth *et al.*, 1986; Everson & Polokoff, 1986), we wondered whether in hepatocytes the weak LDL-mediated down-regulation of the LDL-receptor activity is due to an intracellular diversion of

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; h.p.t.l.c., high-performance thin-layer chromatography.

exogenous LDL-derived cholesterol to a non-regulatory pool.

The present results show that in Hep G2 cells both exogenously delivered (LDL-) cholesterol and endogenously synthesized cholesterol are directed to the cholesteryl ester pool and/or to extracellular cholesterol acceptors, rather than to the regulatory free cholesterol pool involved in regulation of the LDL-receptor activity. Hence our present results offer a possible explanation for the observation that *in vivo* hepatocytes display LDL-receptor activity, notwithstanding the exposure of hepatocytes to physiological concentrations of LDL.

MATERIALS AND METHODS

Materials

Fetal-calf serum and Dulbecco's modified Eagle's medium (cell-culture medium) were obtained from Flow Laboratories (Irvine, Scotland, U.K.). Penicillin, streptomycin and CHOD-PAP-mono-test kit were purchased from Boehringer Mannheim (Mannheim, Germany).

Human serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Silica-gel 60 t.l.c. plates, free cholesterol and Hepes buffer were from Merck (Darmstadt, Germany).

Na ^{125}I (sp. radioactivity 13.3 mCi/ μg) and [7(n)- ^3H]-cholesterol (5 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). [1- ^{14}C]-Oleic acid (57 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Multi-well cell-culture dishes were from Costar (Cambridge, MA, U.S.A.). Compound 58-035 {3-(decyldimethylsilyl)-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide} was generously given by Sandoz (East Hanover, NJ, U.S.A.). Compactin (ML-236B) was a gift from Dr. A. Endo, Tokyo Noko University, Tokyo, Japan. Compound 58-035 and compactin were added from stock solutions in dimethyl sulphoxide so that the final concentration of the latter in the cell cultures was 0.1% (v/v). All other chemicals were reagent grade.

Preparation of serum and lipoproteins

Human serum was prepared from freshly collected blood obtained from healthy donors. Isolation and characterization of LDL and heavy HDL, and the labelling of LDL with ^{125}I , were performed exactly as described by Havekes *et al.* (1986a).

Culturing of Hep G2 cells

The established Hep G2 cell line, derived from human hepatocyte tumour, was obtained from Dr. B. B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA, U.S.A.). The cells were cultured at 37 °C in 25 cm² flasks containing 0.1–0.2 ml of culture medium/cm² supplemented with 10% (v/v) heat-inactivated fetal-calf serum, penicillin and streptomycin, under air/CO₂ (19:1). The medium was renewed twice a week.

At 1 week before the experiment, the cells were treated with trypsin and transferred to 2 cm² or 10 cm² Multiwell dishes with a split ratio of 1:10. At 20 h before the assay, the medium was replaced with culture medium containing 1% human serum albumin and the additions as indicated.

Measurement of the receptor-mediated association of ^{125}I -LDL

Shortly before the experiment, the cells, in 2 cm² Multiwell dishes, were washed three times and incubated with culture medium supplemented with human serum albumin (1%, w/v) for a period of 30 min at 37 °C. The experiment was started by replacing the medium by 0.3 ml of culture medium containing 20 mM-Hepes (pH 7.4), 1% human serum albumin and 10 μg of ^{125}I -LDL/ml in the absence or in the presence of 300 μg of unlabelled LDL/ml. After 3 h of incubation at 37 °C, each cell dish was cooled to 0 °C and the cell-associated LDL was measured exactly as described previously (Havekes *et al.*, 1983). The amount of ^{125}I -LDL that was cell associated after incubation in the absence of unlabelled LDL minus the amount of ^{125}I -LDL that became associated in the presence of unlabelled LDL was taken as 'receptor-mediated cell association'.

Measurement of intracellular free cholesterol and cholesteryl ester content

After the preincubations as indicated, the cells (in 2 cm² wells) were washed four times with phosphate-buffered saline (pH 7.4) containing human serum albumin (1 mg/ml), followed by one wash with phosphate-buffered saline without albumin. Thereafter the cells were harvested by scraping with a rubber policeman and resuspended by sonication (Branson; 60 W, 20 s). Samples (0.1 ml) were taken for measurement of protein content (Lowry *et al.*, 1951). Lipids were extracted from the cell suspension with methanol/chloroform (1:2, v/v), as described by Bligh & Dyer (1959), after addition of cholesterol acetate (2 μg) as an internal standard. The neutral lipids were separated by high-performance t.l.c. h.p.t.l.c. on silica-gel-60 precoated plates. We used the method described by Schmitz *et al.* (1984) with some minor modifications, e.g. the plates were heat-activated (130 °C for 1 h) and pre-run with 10% 1 M-NaOH in methanol and developed in a Camag h.p.t.l.c. apparatus (no. 28520) with the solvent mixture hexane/diethyl ether/propan-2-ol (87:10:3, by vol.). In this developing system, phospholipids and non-esterified fatty acids remained at the origin, whereas free cholesterol, cholesteryl esters, triacylglycerols and the internal standard are completely separated (Fig. 1).

The lipid bands were quantified densitometrically with a Shimadzu CS 910 densitometer, by using the reflection mode at 380 nm. The densitometric responses were integrated automatically by using the Shimadzu data-processor Chromatopac C-RIB.

The validity of the simultaneous determination of free cholesterol and cholesteryl ester by h.p.t.l.c. was evaluated by measuring the h.p.t.l.c. densitometric responses of mixtures of various amounts of free cholesterol and cholesteryl ester. To each mixture a fixed amount of the internal standard was added in order to obtain absolute quantification. The results presented in Fig. 2 show a linear relationship between the amount of lipid applied to the plate and the amount of lipid measured, provided that the amount of lipid applied is between 0.2 and 1 μg for both free cholesterol and cholesteryl ester. From these curves we calculated the respective lipid densitometric response factors. The same mixtures used for this validation of the h.p.t.l.c. technique were used for enzymic determination of free cholesterol and total

Fig. 1. Chromatograph of the separation of neutral lipids extracted from Hep G2 cells on h.p.t.l.c. plates as described in the text

The positions of the neutral lipids are indicated. Phospholipids and non-esterified fatty acids remained on the start position. Abbreviations: FC, free cholesterol; TG, triacylglycerols; IS, internal standard; CE, cholesteryl ester; SQ, squalene.

Fig. 2. Calibration curves of free cholesterol (FC) and cholesteryl ester (CE) separated on h.p.t.l.c. plates

Regression equations and correlation coefficients correspond to the lines for below 1 µg of lipid applied to the plate.

cholesterol. The amount of cholesteryl ester was calculated. Comparison of both methods gave correlation coefficients of 0.99 for free cholesterol and 0.97 for cholesteryl ester.

This h.p.t.l.c. method allows rapid and simultaneous quantification of free cholesterol and cholesteryl esters in tissue cultures with a high degree of sensitivity.

Measurement of cholesterol esterification (ACAT activity)

Cholesterol esterification was measured by determining the incorporation of [^{14} C]oleic acid into labelled

cholesteryl oleate, essentially as described by Goldstein *et al.* (1974). Cells cultured in 10 cm² wells were preincubated for 20 h in culture medium containing 1 % human serum albumin (fatty acid-free) and lipoproteins, compound 58-035 and compactin as indicated in the Figure legends. After preincubation, to 1 ml of incubation medium 10 µl of [^{14} C]oleate/albumin stock solution (10 mM-[^{14} C]oleate, 11 % albumin, 0.25 µCi of [^{14} C]oleate) was added, and cells were incubated for another 2 h at 37 °C. After removing the medium, cells were washed three times with cold Hanks solution, scraped and sonicated. After removal of a 20 µl sample for protein determination and addition of 25000 d.p.m. of [7(n)- ^{3} H]cholesterol as recovery standard, the suspension was extracted as described by Bligh & Dyer (1959). The chloroform layer was evaporated and the residue applied to a thin layer of silica, which was developed in hexane/diethyl ether/acetic acid (80:20:1, by vol.), with cholesterol and cholesteryl oleate as markers. The lipid spots were detected with autoradiography or iodine vapour, scraped off and counted for radioactivity. Recovery of the internal standard was 65–85 %. Under these conditions formation of [^{14} C]-cholesteryl oleate was linear for at least 2 h.

RESULTS

Effect of ACAT inhibitor (compound 58-035) on cellular cholesterol contents and LDL-receptor activity

Fig. 3 shows that incubation of Hep G2 cells with increasing amounts of ACAT inhibitor 58-035 in the presence of 200 µg of LDL/ml resulted in a dose-dependent inhibition of the incorporation of [^{14} C]oleic acid into cholesteryl ester (ACAT activity). Maximum inhibition of about 90 % was reached at a concentration of 5 µg of 58-035/ml.

To investigate whether 58-035 diminishes the accumulation of cholesteryl ester in Hep G2 cells after incubation with LDL, the cells were incubated with increasing amounts of LDL in the absence or presence of 5 µg of 58-035/ml (Fig. 4). The ACAT inhibitor 58-035 inhibited

Fig. 3. Inhibition of cellular cholesterol esterification (ACAT activity) after incubation of the cells for 20 h with increasing amounts of compound 58-035

After preincubation of the cells without 58-035, 0.36 nmol of cholesteryl ester was formed/2 h per mg of cell protein. This value was taken as the control value (100 %). Each value represents the mean of replicate determination.

Fig. 4. Intracellular amounts of free cholesterol, cholesteryl ester and total cholesterol after incubation for 20 h with increasing amounts of LDL in the absence (●) or presence (■) of 5 µg of compound 58-035/ml

Each value represents the mean $\pm$ standard deviation of quadruplicate determinations.

the LDL-induced accumulation of cholesteryl ester substantially (Fig. 4b), whereas the content of free cholesterol did not increase significantly with increasing LDL concentrations in either the absence or the presence of 58-035 (Fig. 4a). Consequently, the total cholesterol content of cells incubated with LDL and ACAT inhibitor was diminished as compared with cells incubated with LDL alone (Fig. 4c). Since the experiment was performed in medium containing human serum albumin instead of lipoprotein-depleted serum, an enhanced efflux of cholesterol from the cells into the medium is not plausible. Therefore these results suggest that 58-035 decreased the cellular uptake of LDL-cholesterol and/or the intracellular cholesterol synthesis. To determine if 58-035 could inhibit the uptake of LDL, we measured the effect of incubation with increasing amounts of 58-035 on the LDL-receptor activity in Hep G2 cells. The results in Fig. 5 show that preincubation of the cells with 58-035 in the presence of 200 µg of LDL/ml resulted in a significant ($P < 0.05$) decrease in LDL-receptor activity at each concentration of 58-035 used. A significant decrease in LDL receptor activity was not found when the cells were preincubated with increasing amounts of 58-035 alone.

To test whether 58-035 enhances the LDL-mediated down-regulation of the LDL-receptor activity, Hep G2 cells were preincubated with increasing amounts of LDL in the absence or presence of 58-035 (5 µg/ml). The ACAT inhibitor significantly ($P < 0.05$) enhanced the LDL-mediated down-regulation at all LDL concentrations used (Fig. 6).

Previously, we reported that in Hep G2 cells and in human hepatocytes (Havekes *et al.*, 1986a,b) the LDL-receptor activity is dramatically increased after incubation of the cells with heavy HDL (density 1.16–1.20 g/ml), possibly owing to a diminished cellular regulatory free cholesterol pool. To investigate whether inhibition of ACAT results in a diminished heavy-HDL-mediated stimulation of the LDL-receptor activity, Hep G2 cells were incubated with increasing amounts of heavy HDL in the absence or presence of 5 µg of 58-035/ml. No

effect of 58-035 on the heavy-HDL-mediated stimulation of the LDL-receptor activity was observed (Fig. 7).

Cellular cholesterol contents and LDL-receptor activity

The observed effect of 58-035 on LDL-mediated down-regulation of the LDL-receptor activity and the absence of an effect of 58-035 on the heavy-HDL-mediated stimulation of the LDL-receptor activity prompted us to study the relationship between intracellular cholesterol contents and LDL-receptor activity (Fig. 8). We measured the cellular contents of free cholesterol (Fig. 8a) and cholesteryl ester (Fig. 8b) after

Fig. 5. Receptor-mediated association of ¹²⁵I-LDL after incubation of the cells for 20 h with increasing amounts of compound 58-035 in the absence (■) or presence (●) of 200 µg of LDL/ml

Associations of ¹²⁵I-LDL to cells incubated in the absence of compound 58-035 were taken as control values (100%). Control values were 44 and 129 ng/mg of cell protein for cells incubated with and without 200 µg of LDL/ml respectively. Each value is the mean $\pm$ s.d. of quadruplicate determinations: *significantly different from that of the same incubation but in the absence of LDL ($P < 0.05$).

Fig. 6. Receptor-mediated association of ^{125}I -LDL after incubation of the cells for 20 h with increasing amounts of LDL in the absence (■) or presence (●) of 5 µg of compound 58-035/ml

Associations of ^{125}I -LDL to cells incubated in the absence of LDL were taken as control values (100%). Control values were 103 and 114 ng/mg of cell protein for cells incubated in the presence or absence of compound 58-035 respectively. Each value represents the mean $\pm$ S.D. of quadruplicate determinations: *significantly different from that of the same incubation but in the absence of compound 58-035 ($P < 0.05$).

Fig. 7. Receptor-mediated association of ^{125}I -LDL after incubation of the cells for 20 h with increasing amounts of heavy HDL in the absence (■) or presence (●) of 5 µg of compound 58-035/ml

The amount of heavy HDL was expressed as the amount of apolipoprotein AI/ml. Each value represents the mean of duplicate determinations.

incubation of the cells with LDL, heavy HDL or a combination of both. These incubations were done in both the presence and the absence of 58-035. Parallel incubations were used for measuring LDL-receptor activity (Fig. 8c).

Under all conditions, the cellular free cholesterol content did not change significantly (Fig. 8a). As shown

Fig. 8. Cellular contents of free cholesterol (a) and cholesteryl ester (b), and receptor-mediated association of ^{125}I -LDL (c), after 20 h incubation of the cells with LDL (200 µg/ml), heavy HDL (200 µg of apolipoprotein AI/ml) or a combination of both

Shaded bars represent incubation in the presence of 5 µg of compound 58-035/ml; '-' represents incubation of the cells in the absence of lipoproteins (control cells). Each bar represents mean $\pm$ S.D. of quadruplicate determinations: *significantly different from each other ($P < 0.05$).

in Fig. 8(b), the cholesteryl ester content increased after incubation with LDL or a mixture of LDL and heavy HDL. Both LDL-dependent increments of cellular cholesteryl ester are inhibited by a simultaneous incubation with 58-035. Incubation of Hep G2 cells with heavy HDL, 58-035 or a combination of both had no significant effect on the cellular content of either free cholesterol or cholesteryl ester (Figs. 8a and 8b).

Incubation of the cells with 58-035 alone (Fig. 8c) did not affect the LDL-receptor activity significantly, as shown in Fig. 5. Incubation of the cells with LDL resulted in a relatively weak (55%), but significant, decrease in LDL-receptor activity, which is further decreased (to 25%) on incubation with 58-035 simultaneously. Heavy HDL stimulated the LDL-receptor activity to about 180–200%, irrespective of whether 58-035 was present or not (Fig. 8c). As compared with incubation with LDL alone, the heavy-HDL-mediated stimulation of the LDL-receptor activity could not be completely prevented by a simultaneous addition of 200 µg of LDL/ml in either the absence or the presence of 58-035.

Fig. 9. Cellular cholesterol esterification (ACAT activity) after incubation of the cells for 20 h with LDL (200 μ g/ml), heavy HDL (200 μ g of apolipoprotein AI/ml) or a combination of both

Shaded bars represent incubation in the presence of 2 μ M-compactin; '-' represents incubation of the cells in the absence of lipoproteins (control cells). Each bar represents mean $\pm$ S.D. of triplicate determinations. Either in the absence or in the presence of compactin, each value is significantly different from the other values ($P < 0.05$).

Cellular cholesterol esterification (ACAT activity)

The LDL-receptor activity is considered to be influenced by the cellular content of free cholesterol. Such a dependency could not be deduced from the results presented in Fig. 8. Therefore, since cholesterol esterification (ACAT activity) is known to be a sensitive measure of excess cellular cholesterol (Brown *et al.*, 1980), we measured the ACAT activity after incubation of the cells with LDL, heavy HDL or a combination of both. In addition, to evaluate whether the intracellular cholesterol synthesis also affects the intracellular cholesterol content, the cells were also incubated with compactin simultaneously. In the absence of lipoproteins, incubation with compactin resulted in about a 75% decrease in ACAT activity (Fig. 9). Incubation of the cells with LDL or a mixture of LDL and heavy HDL led to a 3–4-fold increase in ACAT activity. This increase in ACAT activity was not affected by a simultaneous incubation with compactin. After incubation with heavy HDL in the absence or presence of compactin, the ACAT activity was decreased by more than 95% as compared with the ACAT activity measured in control cells (Fig. 9).

DISCUSSION

The data presented in this paper have demonstrated that Hep G2 cells display ACAT activity, and agree with the results reported by Erickson & Fielding (1986). ACAT can be inhibited by compound 58-035 (Sandoz), as shown by Ross *et al.* (1984). Our results show that in Hep G2 cells a maximum ACAT inhibition was reached at a concentration of 5 μ g of 58-035/ml. We found that 58-035 enhanced the LDL-mediated down-regulation of the LDL-receptor activity in Hep G2 cells (Fig. 6),

whereas incubation with 58-035 alone did not affect this activity (Fig. 5). This suggests that, in the presence of extracellular LDL-cholesterol and 58-035, the intracellular regulatory free cholesterol pool is increased, as compared with incubation with LDL alone. Heavy HDL increased the LDL-receptor activity in Hep G2 cells dramatically (Figs. 7 and 8c), presumably by depletion of free cholesterol from the regulatory free cholesterol pool (Oram, 1983; Havekes *et al.*, 1986a). An effect of 58-035 on the heavy-HDL-mediated stimulation was not observed.

Ross *et al.* (1984) found that in rat hepatoma (Fu5AH) cells compound 58-035 does not affect the incorporation of oleate into phospholipids or diacylglycerols, nor does it inhibit the esterification of retinol. We found that the synthesis of bile acids in rat hepatocytes is also not influenced by the addition of 58-035 (H. M. G. Princen, unpublished work). These results suggest that 58-035 acts rather specifically. Although a direct effect of 58-035 on cholesterol synthesis cannot be excluded, our results suggest that 58-035 does not inhibit the cellular cholesterol synthesis, as in that case incubation of the cells with 58-035 alone would result in an increased LDL-receptor activity. Such an effect of 58-035 on LDL-receptor activity was not observed (Figs. 5 and 8).

Our results prompted us to study the intracellular cholesterol homeostasis in relation to the LDL-receptor activity. Measurements of cellular lipids did not show significant rises in the cellular free cholesterol mass after incubation of the cells either with LDL alone or with LDL plus 58-035 (Figs. 4 and 8a). Similarly, preincubation of the cells with heavy HDL did not result in a significantly diminished cellular free cholesterol mass in either the absence or the presence of 58-035. These results agree with those reported by Tabas *et al.* (1986) as far as the absence of a change in cellular free cholesterol mass after incubation with LDL is concerned, and suggest that an increase or a decrease in the regulatory free cholesterol pool is overshadowed by the far greater amount of free cholesterol present in the plasma membrane. This hypothesis is sustained by the results of Lange & Ramos (1983). They reported that 80% of the cellular free cholesterol mass is present in the plasma membrane and is not in rapid equilibrium with internal membranes. Apparently, a down- or up-regulation of the LDL-receptor activity will be obtained by small changes in the total cellular free cholesterol mass.

ACAT activity has been shown to be a sensitive measure for the excess of free cholesterol content in the regulatory pool (Brown *et al.*, 1980). Therefore, measuring ACAT activity would enable us to measure variations in the regulatory free cholesterol pool, notwithstanding the relatively high amounts of non-regulatory free cholesterol present in the plasma membrane. Indeed, in contrast with an apparent constant cellular free cholesterol mass, the ACAT activity increased dramatically on incubation of the cells with LDL (Fig. 9). Our observation that the ACAT inhibitor 58-035 enhanced the LDL-mediated down-regulation of the LDL receptor in Hep G2 cells suggests therefore that, in Hep G2 cells, as in J774 macrophages (Tabas *et al.*, 1986), the sluggish down-regulation of the LDL-receptor activity (Havekes *et al.*, 1986a) and the cellular cholesterol synthesis (Cohen *et al.*, 1984) by incubation of the cells with LDL is due to a pronounced intracellular diversion of LDL-derived cholesterol to a non-regulatory ACAT-

substrate pool. This is in contrast with the situation in fibroblasts, where the efficient LDL-mediated down-regulation of the LDL-receptor activity and cholesterol synthesis appeared not to be enhanced by 58-035 (Tabas *et al.*, 1986). In addition, it should be emphasized that in liver cells the metabolically active free cholesterol pool is also directed to cholesterol pools for the synthesis of bile acids and VLDL (Suckling & Stange, 1985).

In the absence of endogenously synthesized lipoproteins, cholesterol in the Hep G2 cells is directed to the ACAT substrate pool, since the presence of compactin, an inhibitor of cholesterol synthesis, resulted in a diminished ACAT activity (Fig. 9). On the contrary, in the presence of LDL, compactin did not affect ACAT activity, suggesting that in the presence of LDL the delivery of exogenous cholesterol to the ACAT substrate pool far exceeds the delivery of endogenously synthesized cholesterol to that pool.

Brown *et al.* (1980) reported that, in mouse peritoneal macrophages, cytoplasmic cholesteryl esters undergo a continual cycle of hydrolysis and re-esterification, with a half-life of about 24 h. Incubation of cholesteryl-ester-laden macrophages with cholesterol acceptors such as heavy HDL will disrupt this cycle by removing free cholesterol from the cell, thereby suppressing the ACAT activity and, as a result, decreasing the cellular content of cholesteryl ester. Our results show that, with Hep G2 cells also, incubation of the cells with heavy HDL resulted in a dramatic decrease in ACAT activity, to below the detection limit (Fig. 9). This explains the absence of an effect of 58-035 on the heavy-HDL-mediated stimulation of the LDL-receptor activity (Fig. 7). Furthermore, it indicates that, on incubation with extracellular cholesterol acceptors such as heavy HDL, resulting in an increased hydroxymethylglutaryl-CoA reductase activity, the increased amount of endogenously synthesized cholesterol (Havekes *et al.*, 1986a) is directed to extracellular cholesterol acceptors rather than to the ACAT substrate pool. However, when the cells are incubated with LDL in addition to heavy HDL, exogenously delivered cholesterol is, at least partly, directed to the ACAT substrate pool. After incubation with both LDL and heavy HDL, the increase in cellular cholesteryl ester content is even more pronounced than after incubation with LDL alone (Fig. 8b). This observation must be due to the enhanced LDL-receptor activity on incubation of the cells with a mixture of LDL and heavy HDL as compared with incubation with LDL alone (Fig. 8c).

It is striking that, in contrast with macrophages, suppressing the ACAT activity in Hep G2 cells by incubation with extracellular cholesterol acceptors does not result in a decline in the cellular content of cholesteryl ester during the 20 h of incubation of the cells (Fig. 8b). Furthermore, incubation of Hep G2 cells with ACAT inhibitor 58-035 also did not result in a decline in the amount of cellular cholesteryl ester (Fig. 8b), concomitant with an increase in the free cholesterol content of the cells (Fig. 8a), whereas in macrophages such an effect has been reported (Brown *et al.*, 1980; Schmitz *et al.*, 1985). The reason for the difference between macrophages and Hep G2 cells, with respect to the correlation between ACAT activity on the one hand and cholesteryl ester and free cholesterol contents of the cell on the other, is at present subject to speculation.

In summary, our results strongly suggest that in Hep

G2 cells both exogenously delivered (LDL-) cholesterol and endogenously synthesized cholesterol are primarily directed to the cholesteryl ester pool or, if present, to extracellular cholesterol acceptors (heavy HDL) rather than to the hypothetical regulatory free cholesterol pool involved in regulation of the LDL-receptor activity. To what extent the cellular synthesis and secretion of VLDL (Thrift *et al.*, 1986; Ellsworth *et al.*, 1986) and bile acids (Everson & Polokoff, 1986) are involved in the relationship between cellular cholesterol homeostasis and LDL-receptor activity requires investigation.

Since heavy HDL and apolipoprotein-AI-containing particles are also (artificially) generated during ultracentrifugation (Fainaru *et al.*, 1977; Patsch *et al.*, 1980), it is impossible to determine what proportion of total HDL in plasma is accounted for by the heavy-HDL fraction. However, several reports (amongst others: Slater *et al.*, 1980; Fielding & Fielding, 1981) strongly suggest that low- M_r apolipoprotein-AI-containing particles, which are able to remove cholesterol from the cells, exist in whole human plasma. Provided that the present results obtained with Hep G2 cells hold true for primary cultures of human hepatocytes, our results might explain why *in vivo* hepatocytes display LDL-receptor activity, notwithstanding the exposure of these cells to physiological concentrations of LDL.

We thank Mrs. Lenie de Pagter and Mr. Piet Meyer for culturing Hp G2 cells and excellent technical assistance. Mrs. Clara Horsting-Been and Miss Marisa Horsting are kindly acknowledged for preparing the manuscript.

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Received 9 March 1987/3 July 1987; accepted 30 July 1987