Effect of Apolipoprotein E-free High Density Lipoproteins on Cholesterol Metabolism in Cultured Pig Hepatocytes*

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We studied cholesterol synthesis from [14C]acetate, cholesterol esterification from [14C]oleate, and cellular cholesterol and cholesteryl ester levels after incubating cells with apoE-free high density lipoproteins (HDL) or low density lipoproteins (LDL). LDL suppressed synthesis by up to 80%, stimulated esterification by up to 280%, and increased cell cholesteryl ester content about 4-fold. Esterification increased within 2 h, but synthesis was not suppressed until after 6 h. ApoE-free HDL suppressed esterification by about 50% within 2 h. Cholesterol synthesis was changed very little within 6 h, unless esterification was maximally suppressed; synthesis was then stimulated about 4-fold. HDL lowered cellular unesterified cholesterol by 13–20% within 2 h and promoted the removal of newly synthesized cholesterol and cholesteryl esters. These changes were transient; by 24 h, both esterification and cellular unesterified cholesterol returned to control levels, and cholesteryl esters increased 2–3-fold. HDL core lipid was taken up selectively from [3H]cholesteryl ester- and ether-labeled HDL. LDL core lipid uptake was proportional to LDL apoprotein uptake. The findings suggest that 1) the cells respond initially to HDL or LDL with changes in esterification, and 2) HDL mediates both the removal of free cholesterol from the cell and the delivery of HDL cholesteryl esters to the cell.

Cell-surface binding sites for HDL have been found in cells from a variety of tissues (1–4), and whereas they have similar properties, it is becoming apparent that HDL-cell interactions may serve different functions depending on the kind of cell involved and how it uses cholesterol. At least two general patterns can be discerned in cells from non-hepatic tissues. First, HDL can transport cholesterol into cells from a number of steroidogenic tissues as a precursor for steroid hormone synthesis (5–11). These cells have HDL-binding sites; and when it has been tested, conditions that increase the cell's need for cholesterol also increase the number of HDL-binding sites, suggesting the participation of HDL-binding sites in the delivery of cholesterol to the cells (3, 12, 13). Second, in nonsteroid-secreting cells, the responses of the cells to HDL suggest that this lipoprotein can mediate the removal of cholesterol from the cells. HDL or HDL apoprotein-mediated cholesterol efflux has been observed in a variety of cells including ascites cells (15), fibroblasts (16–19), macrophages (20), and smooth muscle cells. Such cells can esterify and store excess cholesterol but do not metabolize or excrete it. In cases in which it has been investigated, conditions associated with cholesterol accumulation and storage lead to increased high affinity HDL binding (20–23). Furthermore, preincubation with HDL3 stimulates the activity of 3-hydroxy-3-methylglutamyl-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis (24), stimulates LDL receptor activity, suppresses cholesterol esterification (25), and reduces cellular cholesterol content (17, 25). All of these changes suggest the HDL-mediated removal of cholesterol from cells.

There are several mechanisms by which the liver can either accumulate or dispose of cholesterol. Cholesterol can be synthesized de novo or taken up from plasma lipoproteins, esterified for temporary storage, secreted to the plasma on lipoproteins, or excreted to bile as sterol or after conversion to bile acids. High affinity HDL uptake and lysosomal degradation of HDL apoprotein has been observed in several kinds of suspended and cultured hepatocytes and hepatoma cells (26–35), but there are apparently conflicting reports about the influence of HDL on hepatocyte cholesterol metabolism. Several workers found that HDL did not suppress cholesterol metabolism or 3-hydroxy-3-methylglutamyl-CoA reductase activity (36, 37) and did not affect cholesterol esterification or bile acid synthesis in rat hepatocytes (38) or Hep G2 cells (34), suggesting that the lipoprotein did not affect hepatocyte cholesterol metabolism. On the other hand, Drevon et al. (38) reported that HDL cholesterol was esterified in cultured rat hepatocytes. HDL also stimulated cholesterol synthesis in human fetal liver cells (39) and increased LDL receptor activity in cultured pig hepatocytes (26, 40), and high affinity HDL binding was increased in cholesterol-loaded Hep G2 cells (32). These findings suggest that HDL can mediate the removal of cholesterol from hepatocytes. Finally, there is also evidence indicating the selective delivery of HDL cholesteryl esters to the liver both in vivo (41) and in cultured rat hepatocytes (10, 41).

We previously observed the high affinity uptake and lysosomal degradation of apoE-free HDL apoprotein in cultured pig hepatocytes (28) and recently reported that these cells bound HDL reversibly (27). In this study, we have examined the influence of apoE-free HDL on hepatocyte cholesterol metabolism. Our findings indicate that HDL can alter hepatocyte cholesterol homeostasis, resulting in changes in cholesterol synthesis and esterification, primarily the latter, and that these changes occur in response to high affinity HDL binding.
HDL Effects on Hepatocyte Cholesterol Metabolism

**EXPERIMENTAL PROCEDURES**

**RESULTS**

In the following experiments, we examined the influence of apoE-free HDL on several pathways of hepatocyte cholesterol metabolism. For comparison, we also examined the cell's response to LDL. The experiments were conducted as follows. After 24 h in culture, the cells were transferred to serum-free medium for 1 h at 37 °C. Varying concentrations of HDL-L or LDL were then added, and the cells were preincubated at 37 °C for varying periods. At the end of the preincubation, the lipoprotein-containing medium was removed, and we measured the incorporation of [14C]oleate into cholesteryl esters, the incorporation of [14C]acetate into dihydroxipentosyl-precipitable sterols, and the cellular content of unesterified and esterified cholesterol.

Fig. 1A illustrates the effects of preincubating the cells with LDL for varying periods. In the absence of lipoproteins, the absolute level of esterification decreased somewhat, and sterol synthesis increased somewhat between 0 and 27 h. In the presence of lipoproteins, moderate changes occurred to a greater or lesser extent in some, but not all, cell preparations, possibly reflecting differences in the physiological state of the animals at the time the cells were prepared. The patterns observed in response to LDL and HDL, however, were the same regardless of these changes. After a 2-h exposure to LDL (30 or 100 μg/ml lipoprotein cholesterol), esterification was stimulated by 14 and 34%, respectively, compared with cells incubated for the same period without lipoproteins. After 6 h, LDL stimulated esterification by 50 and 90%, and after 27 h, by 94% and 175%, compared with the respective controls. Sterol synthesis was unchanged at 2 h and depressed by 2 and 5% after 6 h and by 46 and 56% after 27 h, compared with the respective controls. Cellular cholesteryl ester mass tended to increase slightly by 6 h at the higher of the two LDL concentrations. There was very little change in cellular unesterified cholesterol.

Fig. 24 shows the effects of preincubating cells for 6 or 24 h with concentrations of LDL up to 200 μg/ml. After a 6-h exposure to LDL, cholesterol esterification increased 2-fold (from 135 to 270 pmol/h/mg of cell protein) (Fig. 24); again, there was little LDL-mediated change in sterol synthesis at this time. After 24 h, esterification increased further. At similar LDL concentrations, the relative increases in esterification shown in Fig. 24 agreed reasonably well with those in Fig. 1A. Both experiments were performed in separate cell preparations. After 24 h, sterol synthesis was suppressed by up to 60% (Fig. 24). Cellular cholesteryl ester mass tended to increase by 6 h and, in this experiment, was 4-fold higher than controls by 24 h; at that time, there was also a slight increase in cellular unesterified cholesterol (Fig. 24). These patterns were the ones expected for cells with functional LDL receptors and are consistent with the net delivery of LDL cholesterol to the cell.

The patterns observed with HDL were more complex. Fig. 1B illustrates the results obtained following preincubation of the cells with HDL-L for periods up to 27 h. After exposure to HDL (30 or 100 μg/ml HDL cholesterol) for 2 h, esterification was reduced by 36 and 53%, compared to the control. Maximal reduction in esterification was observed after 6 h; esterification was reduced by 53 and 79%. Sterol synthesis was only slightly stimulated at the higher HDL level within
much less effective than at 6 h (Fig. 2B). Thus, at 200 µg/ml HDL~L cholesterol, esterification was reduced by about 50%. This level of suppression was obtained at an HDL~L level of 30 µg/ml at the earlier time. In addition, at 200 µg/ml HDL~L, stimulated sterol synthesis by only about 30% compared with almost 4-fold at 6 h. Cellular levels of unesterified cholesterol returned almost to control values, and cholesteryl esters increased by 2-3-fold at the highest HDL~L concentration after 24 h (Fig. 2B). It was noted that the cholesteryl ester content of the cells tended to be higher at the highest HDL level, despite the 50% reduction in esterification under these conditions.

These patterns suggested that HDL facilitated the net removal of cholesterol from the cells during the initial 6 h. After a longer period, the changes were reversed. It was also apparent that cholesterol esterification changed sooner than sterol synthesis and in response to lower concentrations of either HDL or LDL.

In a separate series of experiments, we examined the effect of apoE-free HDL and, for comparison, LDL on cholesterol esterification using preincubation times as short as 30 min. The lipoprotein concentrations used in these experiments ranged from 0 to 30 µg/ml lipoprotein cholesterol.

Fig. 3 illustrates the results obtained when the same two cell preparations were preincubated with HDL~L. A 10-15% reduction in cholesterol esterification occurred within the first 60 min of preincubation with HDL~L (Fig. 4A, left). The reduction reached about 50% within 2 h (Fig. 4A, A, left, and B, left) and 55-65% by 6 h (Fig. 4A, A and B). This suppression was clearly reversed after 24 h (Fig. 4B). There was no change in cellular cholesteryl ester content within the first 6 h (Fig. 4A, A, right, and B, right) or in cellular unesterified cholesterol within the first 60 min (Fig. 4A). There was, however, an approximate 15% decrease in unesterified cholesterol after 2 and 6 h (Fig. 4A, A and B). Again, cell cholesterol content was not measured at 24 h in these experiments.

The HDL-mediated changes in both cholesterol esterification and cholesterol mass occurred predominantly at low concentrations of HDL, suggesting that these effects were mediated through high affinity binding to the HDL-binding site. Since the reduction in esterification increased with time, we considered that high affinity HDL uptake is a time-dependent process that does not reach a plateau until about 8 h (26, 27). We therefore performed an experiment in which both cholesterol esterification and the time course of high affinity HDL uptake at 37°C were measured at the same time. Two sets of plates were used. After 24 h in culture, the first set was transferred to medium that contained 0-45 µg/ml HDL~L cholesterol and was preincubated for 2 or 6 h, and the incorporation of [3H]oleate into cholesteryl ester was then measured as described above. The second set was transferred to 10% LPDS, and the time course of 131I-labeled HDL~L uptake was measured in order to estimate the amount of HDL

the first 6 h. After 27 h of exposure to the lower concentration of HDL, esterification had returned to the level of the control and was suppressed by only about 20% at the higher HDL level. Sterol synthesis at this time was increased by about 37% at the higher HDL concentration. The cell content of unesterified cholesterol was up to 13% lower than the control after 2-6 h, but was not depressed after 27 h. The cellular cholesteryl ester level was essentially unchanged throughout the incubation period.

In order to examine the effect of HDL concentration, the cells were incubated with increasing concentrations of HDL~L for 6 or 24 h. After a 6-h preincubation with HDL~L, there was a marked concentration-dependent reduction of up to 75% in cholesterol esterification (Fig. 2B). Under the conditions of the experiment, half-maximal reduction occurred at a concentration of about 15 µg/ml HDL cholesterol (30 µg/ml HDL protein). In contrast, sterol synthesis increased only slightly (about 30%) at 100 µg/ml HDL cholesterol (Fig. 2B). At 200 µg/ml, however, there was an almost 4-fold increase in sterol synthesis. The cell content of unesterified cholesterol was up to 20% lower after preincubation with HDL~L, and esterified cholesterol was unchanged. As expected from the observations in Fig. 1B, after a 24-h preincubation period, the patterns were different. HDL~L in low concentrations did not suppress esterification at all and at higher concentrations was

Fig. 2. Hepatocyte cholesterol metabolism following preincubation with varying concentrations of lipoproteins. After 24 h in culture, cells were transferred to MEM containing LDL (0-196 µg/ml cholesterol, 0-100 µg/ml LDL protein) (A) or HDL~L (0-200 µg/ml cholesterol, 0-400 µg/ml HDL protein) (B). The cells were incubated for 6 h (upper) or 24 h (lower), after which the lipoproteins were removed, and the incorporation of [3H]oleate into cholesteryl esters and [3H]acetate into cholesterol and cellular unesterified and esterified cholesterol were measured as described under “Experimental Procedures.”
that actually associated with the cells during the preincubation period. Fig. 5A shows the time dependence of $^{125}$I-labeled HDL uptake at concentrations of 3.5 and 9 $\mu$g/ml HDL cholesterol (10 and 25 $\mu$g/ml HDL protein, respectively). At both concentrations, the amount of high affinity cell-associated $^{125}$I-labeled HDL was 1.5-2-fold greater at 6 h compared to 2 h (Fig. 5A). At similar HDL concentrations, the decrease in esterification was about twice as great at 6 h as at 2 h (Fig. 5B). Thus, the greater suppression of esterification at 6 h probably reflected the greater amount of high affinity HDL uptake at 6 h, and the potential rate of suppression may be much greater if not limited by the rate of HDL uptake.

The effects of both HDL L and HDL L, the more dense HDL subfraction (see “Experimental Procedures”), on [14C] oleate incorporation into cholesteryl esters were also examined after preincubating the cells with either of the two subfractions (0-30 $\mu$g/ml HDL cholesterol) for 2, 6, or 24 h. This experiment was performed in parallel with that shown in Fig. 4B. HDL L suppressed esterification by up to 45% within 6 h; and by 24 h, esterification was still reduced by about 20% (not shown). The patterns for HDL L were virtually identical with those shown in Fig. 4B for HDL L, except for a 10-15% increase in esterification at 24 h (not shown). The cholesterol and cholesteryl ester content of the cells following preincubation with HDL L was also the same as for HDL L, (not shown). There was insufficient HDL L available for cellular sterol measurements to be made in response to this HDL subfraction. Thus, the patterns observed with each of the HDL subfractions were similar. Within the first 6 h, there was a marked reduction in esterification, and the decrease was reversed (or for HDL L partially reversed) by 24 h.

The foregoing experiments suggested that apoE-free HDL initially promoted the net removal of cholesterol from the cells, which may have reduced the cellular pool of cholesterol available to be esterified, thus leading to the marked reduction in esterification. The recovery of the cells after long-term preincubation further suggested the operation of one or more mechanisms leading to the re-establishment of cellular cholesterol homeostasis and the restoration of esterification activity. We considered the possibility that the initial HDL-mediated decrease in esterification might have been exaggerated due to the lecithin:cholesterol acyltransferase-mediated esterification of some HDL cholesterol during preparation or storage of the lipoprotein. The resulting unesterified cholesteryl-depleted HDL particle might then have more effectively promoted the loss of cellular cholesterol and magnified the reduction in esterification. We therefore measured cholesteryl esterification following exposure of the cells for 6 h to HDL L (30 $\mu$g/ml HDL cholesterol, equivalent to 88 $\mu$g/ml HDL protein) that had been isolated in the presence or absence of DTNB, a potent inhibitor of lecithin:cholesterol acyltransferase. When isolated in the absence of DTNB, 82.5% of the HDL cholesterol was esterified, compared with 83.3% when DTNB was present. HDL L isolated without DTNB suppressed esterification by 66%, compared with 57% for HDL L isolated in the presence of DTNB (not shown). Thus, neither the cholesterol/cholesteryl ester composition of HDL nor its ability to suppress esterification was changed very much, suggesting that the influence of HDL on the cells had not been exaggerated.

We then examined the influence of HDL L and LDL on the cellular levels of prelabeled cholesterol and cholesteryl esters. The experiments were performed as follows. After the initial period of culture in fetal calf serum (see “Experimental Procedures”), the cells were transferred to MEM, and cellular lipids were labeled endogenously by incubation for 18 h with [14C]acetate. At the end of this period, the labeled acetate was removed, and the cells were transferred to MEM that contained HDL L or LDL (0-50 $\mu$g/ml lipoprotein cholesterol) and incubated for 6 h, after which the radioactivity associated with the cellular lipids was measured as described above (see “Experimental Procedures”). In the absence of lipoproteins, about 9% of the radioactivity associated with the cell sterols was in cholesteryl esters, instead of 18% in unesterified cholesterol. The total amount of [14C]cholesterol, [14C]cholesteryl esters, and [14C]phospholipids recovered from the medium after the 6-h preincubation without lipoproteins was 0.81, 2.73, and 10.11% of the amounts that remained with the cells, respectively. This would represent the maximum amount of these lipids that would have been secreted to the medium from the cells during the 6-h period after the cells were removed from the [14C]acetate-containing medium. There was a reduction of up to about 18% in cell-associated [14C]cholesterol in cells that were incubated with LDL (Fig. 6). [14C]Labeled cholesteryl esters increased somewhat. In contrast, incubation with HDL L resulted in the loss of up to 50% of both the [14C]-labeled cholesterol and cholesteryl esters from the cells (Fig. 6). Neither of the lipoproteins influenced the cellular levels of [14C]labeled phospholipids.

In order to determine the amount and location of cholesterol and cholesteryl esters in the medium following incubation with HDL, an aliquot of the medium from cells that had been incubated with the highest concentration of HDL L (50 $\mu$g/ml HDL cholesterol) was separated into fractions of d < 1.006, d = 1.063-1.21, and d > 1.21 g/ml, and we measured the distribution of [14C]cholesterol and [14C]cholesteryl esters in the three fractions. Ninety-eight percent of the [14C]cholesterol recovered from the medium was unesterified, and 84% of this was in the d 1.063-1.21 g/ml fraction. Another 13%
The direct uptake of unlabeled cholesteryl esters from HDL3L.

by almost 50%. This disparity suggested that the removal of points, presumably reflecting the rapid uptake and lysosomal hydrolysis of ['H]cholesteryl linolate taken up with LDL (Table I). We also compared the flux of LDL cholesteryl esters, measured as the sum of cell-associated ['H]cholesteryl ester and ['H]cholesterol, with the total cellular flux of LDL apoprotein, measured as the sum of cell-associated and degraded ['H]LDL, in order to determine whether the uptake of the core lipid could be accounted for by the rate of apoprotein uptake. The results are shown in Fig. 7A. The flux of LDL apoprotein was 110 ng/h/mg of cell protein. Since the cholesteryl ester:protein ratio of the dual-labeled LDL used for this experiment was 1.40:1, the expected rate of cholesteryl ester uptake would be 154 ng/h/mg of cell protein if the apoprotein was taken up as an intact particle. This value agreed well with the observed value of 175 ng/h/mg of cell protein. Fig. 7A (left) illustrates the correspondence between the expected and observed LDL cholesteryl ester uptake at several time points.

The findings for HDL3L were different. Cell-associated ['H]cholesteryl ester accumulated more rapidly than ['H]cholesterol for the first 9 h (Table I). By 24 h, however, unesterified ['H]cholesterol predominated, suggesting that the hydrolysis of cholesteryl esters delivered by HDL3L had been delayed. The flux of HDL3L apoprotein was 77 ng/h/mg of cell protein. Since the cholesteryl ester:protein ratio of this dual-labeled HDL3L preparation was 0.31:1, the expected rate of HDL3L cholesteryl uptake would be 24 ng/h/mg of cell protein if the lipoprotein was taken up as an intact particle. This value is about one-third the observed value of 74 ng/h/mg of cell protein (Fig. 7A, right).

These measurements suggest that the labeled HDL cholesteryl ester was taken up in preference to HDL apoprotein. It is known, however, that LPDS generally contains some apoA-I. We therefore considered the possibility that some unlabeled apoA-I that may have been present in the LPDS in the medium could have exchanged with labeled apoA-I on the lipoprotein. This would have reduced the apoprotein-specific activity of the dual-labeled lipoprotein, thus reducing the uptake of protein radioactivity and giving an exaggerated impression of selective cholesteryl ester uptake. A similar experiment was therefore performed in which we measured the uptake of dual-labeled HDL3L from serum-free medium. In this experiment, the core lipid was labeled with ['H]cholesteryl hexadecyl ether, a nonhydrolyzable analog of cholesteryl ester. The results are shown in Fig. 7B. Again, the uptake of labeled cholesteryl ester exceeded that which could be accounted for by the uptake of HDL apoprotein. The apoprotein flux was about 74 ng/h/mg of cell protein. The cholesteryl ester:protein ratio of the dual-labeled HDL3L was 0.28:1, and the expected rate of core lipid uptake was therefore about 21 ng/h/mg of cell protein, which is, again, less than one-third the observed value of 67 ng/h/mg of cell protein, as

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**TABLE I**

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Time (h)</th>
<th>Cell-associated ['H]cholesterol (ng/mg cell protein)</th>
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<td>HDL3L</td>
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<tr>
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<td></td>
<td>24</td>
<td>221</td>
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**FIG. 6.** Effect of lipoproteins on prelabeled cellular lipids. Cells were incubated for 18 h in MEM containing 5 mM sodium [14C]acetate (5 μCi/ml) and then transferred to MEM containing LDL (950 μg/ml cholesterol, 0–30 μg/ml LDL protein) or HDL3L (0–50 μg/ml cholesterol, 0–135 μg/ml HDL protein). Cellular lipid-associated radioactivity was extracted, separated by thin-layer chromatography, and measured as described under "Experimental Procedures." The 100% points correspond to 1.500 × 10^6, 1.440 × 10^6, and 1.561 × 10^6 dpm/mg of cell protein for cholesterol, cholesteryl esters, and phospholipids, respectively.
FIG. 7. Uptake of protein- and lipid-labeled lipoproteins. A, after 24 h in culture, cells were transferred to 10% LPDS and incubated with 125I-labeled [3H]cholesteryl linoleate LDL (14 μg/ml LDL cholesteryl ester, 10 μg/ml LDL protein) (left) or 125I-labeled [3H]cholesteryl linoleate HDL3L (3.1 μg/ml HDL choleseryl ester, 10 μg/ml HDL protein) (right) for the times indicated. The lipoprotein cholesteryl ester uptake was measured as the sum of cell-associated plus degraded lipoprotein protein at each time point and the cholesteryl ester:apoprotein ratio of each dual-labeled lipoprotein. The expected values were calculated from measures of cell-associated plus degraded lipoprotein protein at each time point and the cholesteryl ester:apoprotein ratio of each dual-labeled lipoprotein as indicated in the text. B, cells were transferred to serum-free MEM and incubated with 125I-labeled [3H]cholesteryl hexadecyl ether-labeled HDL3L (1.4 μg/ml HDL cholesteryl ester, 5 μg/ml HDL protein) for the times indicated. The curve labeled Expected was calculated as described for A.

estimated from the initial part of the uptake curve in Fig. 7B. Thus, the findings were the same regardless of the presence of LPDS in the incubation medium, indicating that apoprotein exchange did not influence the results significantly under the conditions of the experiments. The observations were consistent with the selective uptake of HDL core lipid.

Finally, because HDL stimulated immediate changes from which the cells recovered after long-term preincubations, we performed an experiment to determine whether this recovery might have resulted from cell-mediated alterations in HDL that may have attenuated its influence on the cells. Since maximal suppression of esterification occurred at 6 h (Figs. 1B and 2B), we examined the effect of preincubation with HDL3L in which fresh HDL3L-containing medium was provided every 6 h. Cells were preincubated in the presence and absence of HDL3L (30 μg/ml lipoprotein cholesterol) for 6, 12, or 24 h, after which the lipoprotein-containing medium was removed, and we measured cholesterol esterification. The medium was changed every 6 h for half the cells that were assayed after 12- and 24-h preincubation periods. The results are shown in Fig. 8.

Esterification was reduced to 65% of the control level after the first 6 h. By 12 h, esterification began to recover and was about 82% of the control value regardless of whether the medium had been changed at 6 h. After 24 h of preincubation with HDL3L, esterification had returned to the level of the control in the cells in which the medium had not been changed. Changing the medium every 6 h increased the absolute rate of esterification by about 60% in both the controls and the cells which had been exposed to HDL3L; the rate of esterification in the latter was 83% of that in the controls.

FIG. 8. Effect of periodic medium change on cholesterol esterification in pig hepatocytes. After 20 h in culture, cells were transferred to MEM and preincubated in the presence or absence of HDL3L (30 μg/ml cholesterol, 83 μg/ml HDL protein) for the times indicated. Parallel sets of plates were used at 12 and 24 h. In one set, the medium was not changed during the preincubation; and in the other, the medium was changed every 6 h. At the end of each preincubation period, cholesterol esterification was measured as described under "Experimental Procedures."

We previously described the presence of high affinity binding sites for apoE-free 125I-labeled HDL in cultured pig hepatocytes (26). We found that binding to these sites had both reversible and degradative components in which about two-thirds of the cell-associated HDL was subsequently released back to the medium as a somewhat denser lipoprotein. The remaining one-third was internalized and degraded in lysosomes (27). These findings suggested that HDL might be capable of delivering cholesterol to the cells directly through the reversible or degradative components of binding, or both. If so, the resulting changes in cellular cholesterol homeostasis would expectedly be reflected by changes in cell cholesterol metabolism.

In this study, we found that both LDL and apoE-free HDL altered cholesterol homeostasis in pig hepatocytes. For LDL, the patterns of biochemical changes suggest that this lipoprotein mediated the one-way movement of cholesterol into the cells. Thus, after incubation with LDL, cholesterol esterification was stimulated, cholesterol synthesis was suppressed, and there was an increase in the cell content of cholesteryl esters. These changes occurred at low concentrations of LDL and were expected since LDL catabolism is mediated primarily by LDL receptors in cultured pig hepatocytes (27). The experiments also revealed that changes in cholesterol esterification occurred faster than changes in sterol synthesis, inasmuch as the stimulation of esterification began to be evident after 2 h, whereas sterol synthesis was not appreciably suppressed until after 6 h. These observations suggest that...
the cells' initial compensating response to the increased cholesterol load was an increase in cholesterol esterification.

In contrast, the patterns we observed when the cells were preincubated with apoE-free HDL suggested that HDL mediated the two-way movement of cholesterol into and out of the cells. Thus, within 2 h, there was a marked reduction in esterification, accompanied by a 13–20% reduction in cellular unesterified cholesterol content. Indeed, a slight reduction in esterification was detected within 30 min, although there was no detectable decrease in unesterified cholesterol within this period.

The rapid reduction in esterification was apparently mediated by high affinity HDL-binding sites, first, because the reduction was concentration-dependent and occurred primarily at low HDL concentrations at which high affinity binding predominates (26, 27), and second, because the extent to which esterification decreased was related to the rate of high affinity HDL uptake. As with LDL, cholesterol esterification changed before sterol synthesis. This was particularly evident from the data in Fig. 2B. There was no much change in sterol synthesis within 6 h, except at the highest HDL level at which esterification was maximally suppressed. At this point, sterol synthesis was stimulated severalfold.

It should be mentioned that the base-line levels of both cholesterol esterification and cholesterol synthesis generally tended to change somewhat in the absence of lipoproteins as the preincubation time increased. The magnitude of the changes was variable, and they were not observed in all cell preparations. Inasmuch as the experiments were performed within the first 24 h in culture, the changes may have been related to residual hormonal or other physiological factors affecting the metabolic state of the liver at the time the cells were isolated. The patterns of cellular responses to the lipoproteins, however, were the same regardless of whether or not absolute changes occurred in the base-line levels of cholesterol esterification and synthesis.

The observations with HDL	extsubscript{3L} were consistent with the HDL-mediated removal of cholesterol from the cells. This interpretation was supported by the findings in cells in which cholesterol, cholesteryl esters, and phospholipids had been endogenously prelabeled with [14C]acetate. HDL	extsubscript{3L} promoted the loss of up to half of each labeled sterol during a 6-h period, and most of the label released to the medium was recovered in the density range above 1.063 g/ml; only about 3% was in the fraction of d < 1.063 g/ml. These losses were HDL	extsubscript{3L} concentration-dependent and were specific for sterol since there was no significant change in cellular levels of labeled phospholipids under these conditions. In contrast, there was a less than 20% loss of [14C]cholesterol in the presence of LDL, and some of this was apparently converted to cholesteryl esters in response to the influx of cholesterol with LDL since the amount of label in cholesteryl esters increased somewhat in the presence of LDL. Again, LDL did not significantly affect the levels of labeled phospholipids.

For several reasons, it is likely that HDL mediated the loss of sterol in the form of free cholesterol. First, measurements in rat hepatocytes have indicated that about 80% of the cell cholesterol is present as unesterified cholesterol associated with the plasma membrane (52). Second, it is known that unesterified cholesterol can be transferred from the cell surface to various acceptors in the medium, including HDL and apo-HDL-phospholipid complexes (25, 53, 54). Third, apoE-free HDL can bind reversibly to cultured pig hepatocytes (27), which has the effect of concentrating the lipoprotein at the cell surface temporarily, and would be expected to facilitate the movement of cholesterol from the cell membrane. Finally, in this study, the finding that only 2% of the sterol-associated 14C in the medium was in the esterified sterol fraction following preincubation of prelabeled cells with HDL	extsubscript{3L}, compared with 9% in the cell-associated cholesteryl ester fraction, suggests that intracellular cholesteryl esters may have been hydrolyzed before being removed from the cell. This conclusion is tentative, however, because in absolute terms (dpm/mg of cell protein), the amount of labeled cholesterol lost from the cells was 10-fold greater than the amount of cholesteryl esters lost (see legend to Fig. 6), leading to some degree of uncertainty in the measurements.

Considered together, the foregoing observations suggest first, that HDL initially mediated the net removal of cholesterol, perhaps through reversible HDL binding, and second, that the cells responded to the loss with a reduced rate of cholesterol esterification and increased cholesterol synthesis. Esterification changed much sooner than cholesterol synthesis, but the reason for this difference is unclear. Cholesterol synthesis may not have been affected at all until esterification could be suppressed no further. Alternatively, the cell may have responded to the loss of cholesterol by simultaneously beginning to down-regulate esterification and to stimulate cholesterol synthesis, but the esterification pathway may simply have responded faster. It is of interest that Brinton et al. (55) recently reported that high affinity HDL-binding sites mediated cholesterol efflux from cultured fibroblasts.

These changes, however, were transient. The biochemical patterns observed after 24 h were consistent with the re-establishment of cholesterol homeostasis. First, at low HDL concentrations, the inhibition of esterification was clearly reversed and perhaps even slightly stimulated. Second, even at high HDL concentration, where the reduction in esterification and stimulation of sterol synthesis were still evident at 24 h, both of these changes were clearly less pronounced than at 6 h. Third, the cellular content of unesterified cholesterol had almost returned to control levels by 24 h, and there was an increase in the cellular cholesteryl esters, even though cholesterol esterification itself had not yet returned to control levels at higher HDL concentrations.

A number of factors, alone or in combination, might account for the apparent restoration of cholesterol homeostasis during long-term preincubations with HDL. First, it is known that cultured rat hepatocytes (56) and human Hep G2 cells (57) synthesize apoB-containing lipoproteins in culture, and we have found that cultured pig hepatocytes synthesize apoB at a rate of about 40–60 ng/b/mg of cell protein during the period in which these experiments were performed.3 Cholesterol uptake might therefore be mediated in part through the transfer of HDL cholesterol to apoB-containing lipoproteins secreted to the medium, followed by uptake through the LDL receptor. Second, although we have not measured it, the cells most likely also secreted apoE, which might accumulate in the medium to a sufficient extent during long-term incubations to affect the uptake of the HDL	extsubscript{3L} particle, perhaps by directing its uptake through the LDL or apoE receptors. Third, the cells may also secrete enzymes such as hepatic lipase or lecithin:cholesterol acyltransferase. Treatment of HDL with hepatic lipase has been reported to stimulate the net uptake of HDL cholesterol by rat hepatoma cells by a mechanism that did not require the uptake and degradation of the entire HDL particle (58). Lecithin:cholesterol acyltransferase secreted to the medium might enhance the removal of cellular unesterified cholesterol by esterifying HDL unesterified cholesterol, thereby producing a relatively cho-

lesterol-depleted particle which would be a better acceptor for cellular unesterified cholesterol. On the other hand, if HDL cholesteryl esters are taken up preferentially (see below), lecithin:cholesterol acyltransferase-mediated enrichment of HDL cholesteryl esters might increase the delivery of sterol to the cells. Fourth, HDL unesterified cholesterol has been suggested as a principal precursor for bile acid synthesis (59, 60). The additional demand for cholesteryl for bile acid synthesis might also stimulate cholesteryl uptake from HDL. It might be mentioned, however, that bile acid synthesis in cultured rat hepatocytes was reported to be regulated by cellular cholesterol availability (36); and although bile acid synthesis was stimulated by very low density lipoproteins in rat hepatocytes and perfused rat liver, synthesis was unaffected by LDL or HDL in either system (36, 62). Finally, HDL might also deliver cholesteryl esters directly to the cells in a manner similar to that reported by others in rat hepatocytes (10, 42). In those studies, labeled cholesteryl ether was taken up from HDL in amounts exceeding those that could be accounted for by the uptake of intact HDL particles, suggesting that HDL core lipid was taken up preferentially.

Thus, during long-term preincubations with HDL, a number of different mechanisms, alone or in combination, may have operated to reverse the initial HDL-mediated biochemical changes. Several of these mechanisms involve possible cell-mediated changes in HDL during prolonged incubation, and the recovery of the cells might be expected to be prevented under conditions in which the medium was changed frequently to obviate the accumulation of cell-modified HDL. Replacement of the preincubation medium did not prevent the initial recovery of esterification after 12 h, but apparently resulted in incomplete recovery after 24 h. Thus, cell-mediated modification of HDL may have contributed to the cells’ recovery, but other mechanisms probably also operated. In this work, we also examined the possible direct delivery of HDL cholesteryl esters to the cells. We found that labeled cholesteryl ester was taken up directly from dual-labeled HDL and accumulated in the cells at least 9 h. By 24 h, the rate of increase of labeled cholesteryl ester was lower, and more label was associated with unesterified cholesterol, suggesting an initial delay in the hydrolysis of cholesteryl esters delivered by HDL. The reason for this delay is unknown at present. In contrast, cholesteryl ester delivered with dual-labeled LDL was rapidly hydrolyzed.

We also found that the rate of uptake of core lipid from dual-labeled HDL was considerably greater than could be accounted for by HDL apoprotein uptake. These findings suggest the preferential uptake of HDL cholesteryl ester. The mechanism for selective cholesteryl ester uptake is unknown; but in view of the reversibility of HDL binding (27), it may involve the reversible component of binding to HDL-binding sites. It might be conjectured that the direct uptake of cholesterol from HDL, perhaps in the form of cholesteryl ester, may function in vivo and contribute to the return of cholesterol from peripheral tissues to the liver. Such a mechanism might be expected to assume proportionately greater importance in species such as the pig, which has little if any circulating cholesteryl ester transfer protein to mediate the movement of cholesteryl esters from HDL to the lower density lipoproteins (63).

It should be noted that the apparent preferential uptake of HDL cholesteryl esters was clearly evident at the earliest time point. Since the HDL-mediated reduction in cellular unesterified cholesterol is also evident after a relatively short time, we feel it likely that both the inward movement of cholesteryl ester and the outward movement of unesterified cholesterol occurred simultaneously. The biochemical measurements probably reflected the sterol balance of the cells at the time the measurements were made; but as discussed above, this balance may have also been influenced by mechanisms other than those we studied.

It is not clear how HDL reduced the rate of cholesterol esterification, but several possible mechanisms might be mentioned. First, the cellular level of acyl-cholesterol acyltransferase might be altered due to changes in the rate of enzyme synthesis or degradation. The effect was detectable within 30 min, however, which is probably too rapid to be accounted for by this mechanism. Second, acyl-cholesterol acyltransferase activity might be altered by changes in the amount of available cholesterol or by some other activation-inactivation mechanism. Third, cholesteryl-ester hydrolase activity could be increased and thereby prevent the accumulation of cholesteryl esters. These questions are currently being addressed in ongoing studies in our laboratory.

The findings of this study lend further support for the proposed role of HDL in reverse cholesterol transport in vivo. Such a mechanism could account for a significant amount of cholesterol delivery to the liver. For example, it can be calculated that HDL contributes about 900 mg of esterified cholesterol to the circulation in the adult pig (14, 64), or about 20% of the total circulating sterol nucleus. It can be estimated that the liver removes about 9% of the plasma pool of apoAI/h in the rat in vivo (41). Assuming a similar rate in the pig, the liver would be expected to remove about 81 mg of esterified cholesterol/h if HDL is cleared as an intact lipoprotein particle and about 300 mg/h assuming a 3–4-fold greater delivery of esterified cholesterol than HDL apoprotein. If the cholesterol content of pig liver is similar to that in humans (61), this would amount to the hourly delivery of about 6 or 7% of the total liver cholesterol content.

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REFERENCES
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...drops of glacial acetic acid, and ethanol was precipitated with 4 ml 0.1 M sodium bi-carbonate in 50 ml ethanol. The precipitate was washed with acetonitrile (1:1 v/v), dried under N₂, and radioactivity was measured by liquid scintillation spectrometry. Cholesterol synthesis is expressed in percent [¹⁴C]acetate incorporated into cholesterol/mg cell protein. In several experiments, the reproducibility of the data was assessed through the entire analyzation procedure to estimate the reproducibility of the assay. The difference between duplicate assays was about 15% of the mean value.

Figure 1: Effect of HDL on cholesterol esterification. Two different cell preparations were used. In the first (left panels) cells were incubated in HDL-containing DMEM for 10 min and 60 min, 24 h. After which cholesterol esterification and cellular cholesterol and cholesterol ester content were measured as described in Methods. The data in A and B were obtained in the same two hepatocyte preparations used in Fig. 2. The experiments in A and B were performed in parallel with data in Fig. 2 (right panels). HDL-cholesterol concentration ranged from 0 to 150 mg/ml, equivalent to 10 μg/ml HDL-cholesterol.