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Differential uptake and metabolism of free and esterified cholesterol from high-density lipoproteins in the ovary

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Rat luteal cells utilize high-density lipoproteins (HDL) as a source of cholesterol for steroid synthesis. Both the free and esterified cholesterol of HDL are utilized by these cells. In this report, we have examined the relative uptake of free and esterified cholesterol of HDL by cultured rat luteal cells. Incubation of the cells with HDL labeled with [^{3}H]cholesterol or [^{3}H]cholesteryl linoleate resulted in 4–6-fold greater uptake of the free cholesterol compared to esterified cholesterol. The increased uptake of free cholesterol correlated with its utilization for progestin synthesis: utilization of HDL-derived free cholesterol was 3–6-fold higher than would be expected from its concentration in HDL. The differential uptake and utilization of free and esterified cholesterol was further examined using egg phosphatidylcholine liposomes containing cholesterol or cholesteryl linoleate as a probe. Liposomes containing free cholesterol were able to deliver cholesterol to luteal cells and support steroid synthesis in the absence of apolipoproteins, and the addition of apolipoprotein A-I (apo A-I) moderately increased the uptake and steroidogenesis. Similar experiments using cholesteryl linoleate/egg phosphatidylcholine liposomes showed that inclusion of apo A-I resulted in a pronounced increase in the uptake of cholesteryl linoleate and progestin synthesis. These experiments suggest that free cholesterol from HDL may be taken up by receptor-dependent and receptor-independent processes, whereas esterified cholesterol uptake requires a receptor-dependent process mediated by apolipoproteins.

Introduction

Rat luteal cells utilize HDL-derived cholesterol as a substrate for steroidogenesis and are dependent on lipoprotein cholesterol for maximal steroid output [1–4]. Previous studies from this laboratory have demonstrated the presence of specific receptors for HDL in luteal cell plasma membranes which are inducible with tropic hormones [5,6]. In HDL, about 80% of the cholesterol is present as esters and the remainder is free cholesterol. Although our previous studies demonstrated that both free and esterified cholesterol are taken up and utilized for steroid synthesis, it is not clear whether they are taken up in the same proportion as they exist in HDL [7]. This information would be important, since in nonsteroidogenic tissues, it has been reported that HDL removed cellular cholesterol by exchange of free cholesterol be-

Abbreviations: CG, chorionic gonadotropin; apo A-I, apolipoprotein A-I.

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tween the cell membrane and the HDL particle, the equilibrium of which is determined by their respective cholesterol concentrations. In the plasma, esterification of HDL free cholesterol by lecithin–cholesterol acyltransferase could shift this equilibrium to favor transfer of cholesterol from the cell membrane to HDL and facilitate the efflux of cellular free cholesterol [8]. However, steroidogenic tissues such as the ovary utilize HDL cholesterol for steroid synthesis. In these cells, it is possible that transfer of free cholesterol from HDL to the cells will be the favored direction of cholesterol flux. If such a process exists in luteal cells, it will be complementary to the receptor-mediated uptake and utilization for steroidogenesis of free and esterified cholesterol. In this study, we have examined this aspect of HDL metabolism in cultured rat luteal cells. Our results indicate that the free cholesterol is taken up by receptor-mediated and receptor-independent processes, whereas esterified cholesterol uptake requires a receptor-dependent process mediated by apolipoproteins.

Materials and Methods

Materials. Collagenase (type CLS) was the product of Cooper Biomedicals, Malvern, PA. McCoy's medium, fetal bovine serum and horse serum were from GIBCO, Grand Island, NY. Egg phosphatidylcholine, cholesterol, cholesteryl linoleate, pregnant mare serum gonadotropin and human chorionic gonadotropin (CG) were obtained from Sigma, St. Louis, MO. [1,2,6,7-^{3}H]Cholesterol (70 Ci/mmol), [1,2,6,7-^{3}H]cholesteryl linoleate (75 Ci/mmol) and Na^{125}I were purchased from New England Nuclear, Boston, MA. 10% agarose gel (Bio-Gel A-0.5 m) was from Bio-Rad Laboratories, Richmond, CA, and plastic silica gel G plates for thin-layer chromatography were purchased from Eastman Kodak, Rochester, NY. Bicinchoninic acid protein assay reagents were from Pierce Chemical Co., Rockford, IL. All other chemicals were purchased from Fisher Scientific, Detroit, MI.

Animals and cell culture. Luteal cells from the highly luteinized ovaries of immature rats primed with pregnant mare serum gonadotropin and human CG were dispersed with collagenase and cultured in 35 mm plastic tissue culture dishes (Falcon Plastics, Oxnard, CA) in serum supplemented McCoy's medium (comprising 10% horse serum/2.5% fetal bovine serum/50 μg/ml gentamicin) as described previously [9]. Each culture dish contained the equivalent of 600–700 μg cellular protein. Before conducting the experiments described here, the serum-supplemented medium was removed and the cells were washed 4 times with McCoy's medium.

Lipoproteins and labeling. Human HDL_3 (density 1.125–1.21 g/ml) was isolated from plasma of healthy donors by sequential ultracentrifugation after density adjustment with KBr, as described by Havel et al. [10]. Absence of apolipoprotein E in the HDL was confirmed by SDS-polyacrylamide gel electrophoresis. Throughout this paper, quantities of HDL are expressed in terms of its protein content. The ratio of free cholesterol/esterified cholesterol/protein in the HDL was 1 : 5.9 : 51.4. HDL was labeled with ^{125}I by the iodine monochloride method of McFarlane [11] with some modifications [5] to a specific activity of 300–500 cpm/ng protein. Labeling with [^{3}H]cholesterol was accomplished by a modification of the procedure of Jonas et al. [12] as previously described [7]. The specific activity of the labeled HDL was 14 000 cpm/μg of free cholesterol for [^{3}H]cholesterol-labeled HDL and 19 000 cpm/μg esterified cholesterol for cholesteryl linoleate-labeled HDL.

Egg phosphatidylcholine liposomes. Apolipoprotein A-I (apo A-I) was isolated from HDL by the procedure of Scanu and Edelstein [13] as follows: HDL was delipidated with ice-cold ethanol/diethyl ether (1 : 2), the protein residue was dissolved in 6 M urea and chromatographed on a 1.6 × 90 cm Sephacryl S-200 column and eluted with 6 M urea. Fractions containing apo A-I were pooled and dialyzed against McCoy's medium. Purity of the apo A-I was checked by SDS-polyacrylamide gel electrophoresis and was found to be homogeneous by Coomassie blue staining.

Egg phosphatidylcholine vesicles containing cholesterol or cholesteryl linoleate were prepared by sonication of the components according to the procedure of Roth et al. [14] as previously reported from this laboratory [6]. When apo A-I was to be included in the liposomes, the sonicated

preparation was mixed with the apo A-I and incubated overnight at room temperature as described [6]. Cholesterol contents of the liposomes were determined enzymatically.

Determination of HDL cholesterol uptake and utilization for steroid synthesis. To determine the uptake of HDL-associated free cholesterol, cell cultures were incubated in quadruplicate in 1 ml of McCoy's medium containing [^{3}H]cholesterol-labeled HDL (50–300 μg) at 37°C for various time periods (10 min–4 h). For the determination of cholesteryl ester uptake, parallel incubations using HDL containing [^{3}H]cholesteryl linoleate were also performed. The HDL used in both cases had identical chemical composition. At the end of the incubation, the cells were washed four times with McCoy's medium to remove unbound HDL. The cells were extracted from the dishes with 0.1 M NaOH (2 extractions, 0.75 ml each), treated with 50 μl of glacial acetic acid followed by 10 ml of scintillation fluid and counted to determine the amount of [^{3}H]cholesterol or [^{3}H]cholesteryl linoleate taken up. pmol free and esterified cholesterol taken up were calculated from these results and from the specific activity of the labeled sterol in HDL.

For determination of the utilization of HDL-derived free and esterified cholesterol for steroid synthesis, the cells were incubated in quadruplicate with HDL containing [^{3}H]cholesterol or [^{3}H]cholesteryl linoleate as described above. At the end of the incubation period, the cells were washed four times with McCoy's medium and then subjected to a second incubation at 37°C in 1 ml medium containing 100 ng human CG. After 3 h, the medium, containing released steroids, was collected and an aliquot (100 μl) was mixed with scintillation fluid and counted to determine the amount of total radioactivity released into the medium. The remainder was treated with 50 μl of a solution of carrier steroids in benzene (containing 20 μg each of cholesterol, cholesteryl linoleate, progesterone and 20-α-hydroxy-4-pregnen-3-one) and extracted with chloroform/methanol (2:1), according to the procedure of Bierman et al. [15]. The organic layer was separated, dried down under nitrogen, and the residue was redissolved in 100 μl of chloroform. The chloroform extract was subjected to thin-layer chromatography on silica gel plates using a solvent system of petroleum ether/diethyl ether/acetic acid (75:25:1). The spots were visualized with iodine vapor, excised and counted after adding scintillation fluid. The concentrations of labeled steroids, cholesterol and cholesteryl esters in the incubation medium were calculated from these counts and the total amount of radioactivity present in the incubation medium.

Binding assays. The procedure for ^{125}I-labeled HDL binding assay has been described previously by workers from this laboratory [5]. Briefly, cultured cells were incubated for 3 h at 37°C with 1 μg of ^{125}I-labeled HDL in the absence and presence of unlabeled HDL (300 μg) or phosphatidylcholine liposomes (300 μg phosphatidylcholine with varying amounts of apo A-I, cholesterol or cholesteryl linoleate). The cells were washed four times, extracted with 1.5 ml of 0.1 M NaOH and the amount of ^{125}I-labeled HDL bound was determined.

The procedure for [^{3}H]cholesterol- and [^{3}H]cholesteryl linoleate-labeled phosphatidylcholine liposome uptake assays was similar to that used for ^{125}I-labeled HDL binding. The incubation medium contained 300 μg phosphatidylcholine/ml, 15 μM [^{3}H]cholesterol or [^{3}H]cholesteryl linoleate, and varying amounts of apo A-I.

Determination of steroid synthesis in the presence of egg phosphatidylcholine liposomes. Luteal cell cultures were incubated in triplicate at 37°C in 1 ml of McCoy's medium with egg phosphatidylcholine liposomes (300 μg) containing cholesterol or cholesteryl linoleate (0–100 μM final concentration) and apo A-I (0–300 μg) and human CG (100 ng). At the end of the 3 h incubation, the medium was collected, heated in a boiling water-bath for 5 min, centrifuged at $3000 \times g$ for 10 min, and the supernatant was used for determination of progesterone and 20-α-hydroxy-4-prenen-3-one by radioimmunoassay.

Other analytical procedures. Protein contents of samples were determined by the bicinchoninic acid procedure [16]. Cholesterol and cholesteryl esters were assayed by the method of Deacon and Dawson [17]. The data were analyzed by analysis of variance and Duncan's multiple range test. A value of $P < 0.01$ was considered significant. Results are also expressed as mean ± S.E. where indicated.

Results

The uptake of cholesterol and cholesteryl esters from HDL by luteal cells was examined in the experiment shown in Fig. 1A. Cell cultures were incubated in parallel with 300 μg of either [^{3}H]cholesterol-labeled HDL or [^{3}H]cholesteryl linoleate-labeled HDL. Both of the labeled HDL preparations had the same chemical composition, and the ratio of free cholesterol/esterified cholesterol was 1 : 5.9. At various time points up to 4 h, the cell-associated radioactivity was determined and the amounts of free and esterified cholesterol taken up by the cells were calculated. The 'theoretically expected cholesterol uptake' shown in Fig. 1 (and Fig. 2) is the amount of free cholesterol that would have been taken up if free and esterified cholesterol were taken up in the same proportion as they are present in HDL. These values were calculated from the ratio of free to esterified cholesterol in HDL (1 : 5.9) and the amount of cholesteryl ester taken up. The experimentally determined amount of free cholesterol taken up was 5–6-times greater than the expected amount at all the time points examined, resulting in a nearly 1 : 1 ratio of free and esterified cholesterol uptake. The uptake of cholesterol was not linear during the 4 h of incubation.

After having found that the uptake of free cholesterol is 5–6-times greater than expected, we

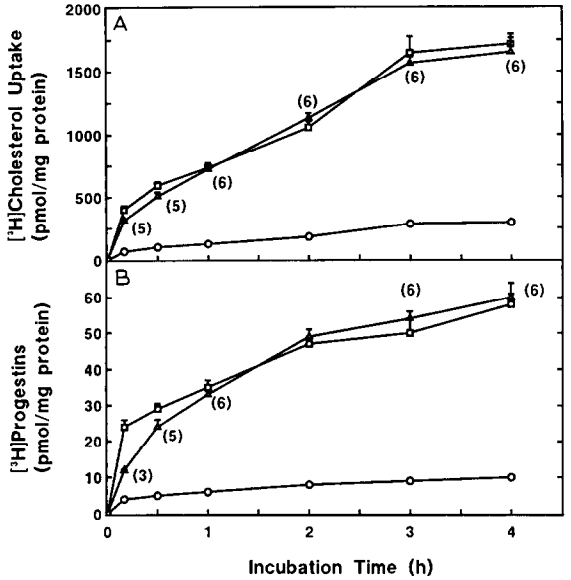

Fig. 1. Uptake and utilization for steroid synthesis of free and esterified cholesterol from HDL by cultured rat luteal cells. (A) Luteal cell cultures were incubated in parallel with HDL (300 μg) labeled with either [^{3}H]cholesterol or [^{3}H]cholesteryl linoleate and the cell-associated radioactivity at various time points was determined (B) Cell cultures were incubated with labeled HDL as in the upper panel, washed and subjected to a second incubation (3 h) in the presence of 100 ng of human CG. The amount of [^{3}H]progestins released into the medium was quantitated by thin-layer chromatography. □———□, cholesteryl ester uptake/utilization; △———△, free cholesterol uptake/utilization; ○———○, theoretically expected amount of free cholesterol uptake/utilization. Numbers in parentheses indicate the fold increase of free cholesterol uptake/utilization over expected amounts. Values are mean ± S.E. of quadruplicate determinations.

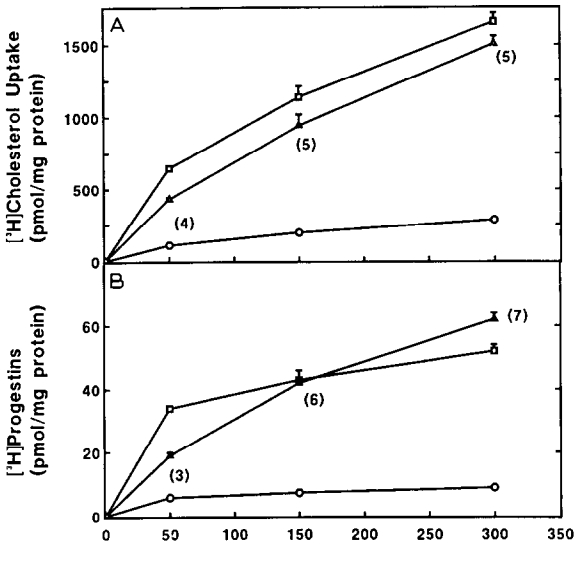

Fig. 2. Effect of HDL concentration on the uptake and utilization of free and esterified cholesterol. (A) Luteal cell cultures were incubated for 4 h with the indicated concentration of HDL labeled with [^{3}H]cholesterol or [^{3}H]cholesteryl linoleate and the amount of labeled sterol taken up was determined. (B) Cell cultures were incubated with labeled HDL as in the upper panel, washed and reincubated for 3 h with 100 ng of human CG, and the amount of [^{3}H]progestins released into the medium was quantitated. □———□, cholesteryl ester uptake/utilization; △———△, free cholesterol uptake/utilization; ○———○, theoretically expected amount of free cholesterol uptake/utilization. Numbers in parentheses indicate the fold increase of free cholesterol uptake/utilization over expected amounts. Values are mean ± S.E. of quadruplicate determinations.

proceeded to determine whether this difference is reflected in the utilization of HDL-derived cholesterol for steroid synthesis. Luteal cells were incubated with labeled HDL for various time periods, as described above. The cells containing labeled HDL were subjected to a second 3 h incubation in the presence of human CG, and the amount of [^{3}H]progestins produced was assayed. The results (Fig. 1B) show that incorporation of [^{3}H]cholesterol into progestins was also 5–6-fold higher than would be expected from its concentration in the HDL: about one-half of the total labeled progestins were derived from the free [^{3}H]cholesterol.

In the experiments shown in Fig. 2, we have examined the effect of HDL concentration on the uptake of [^{3}H]cholesterol and [^{3}H]cholesteryl linoleate from labeled HDL, and the utilization of these compounds for steroid synthesis. Cells were incubated for 4 h with the indicated concentration of HDL labeled with [^{3}H]cholesterol or [^{3}H]cholesteryl linoleate and the amount of label taken up was determined (Fig. 2A). It was found that, irrespective of the HDL concentration, the amount of cholesterol uptake was 5–6-fold higher than that expected from the ratio of cholesterol to cholesteryl esters in HDL. Incorporation of HDL cholesterol into progestins also followed a similar pattern (Fig. 2B); an approx. 5-fold greater incorporation of HDL free cholesterol compared to cholesteryl esters was observed.

The foregoing experiments suggest the existence of different mechanisms for the uptake of free and esterified cholesterol from HDL in luteal cells. We reasoned that the preferential uptake and utilization of unesterified cholesterol from HDL may be due to its receptor-independent uptake (by exchange with the cell membrane), whereas esterified cholesterol will be utilized only through a receptor-mediated process. This possibility was investigated by determination of the effect of apo A-I, the major protein component of HDL, on the uptake of cholesterol or cholesteryl linoleate incorporated into egg phosphatidylcholine liposomes by luteal cells. In the experiment shown in Fig. 3, we examined whether egg phosphatidylcholine liposomes compete with ^{125}I-labeled HDL binding to luteal cells. Cell cultures were incubated for 3 h with 1 μg of ^{125}I-labeled

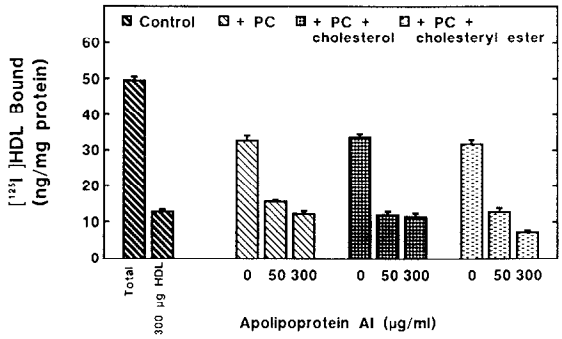

Fig. 3. Inhibition of ^{125}I-labeled HDL binding by egg phosphatidylcholine liposomes. Luteal cell cultures were incubated for 3 h with 1 μg/ml ^{125}I-labeled HDL in the absence and presence of 300 μg unlabeled HDL or egg phosphatidylcholine liposomes (300 μg phosphatidylcholine/ml) and the amount of cell-bound ^{125}I-labeled HDL was determined. Values are mean ± S.E. of triplicate determinations.

HDL in the absence and presence of excess unlabeled HDL or various liposome preparations. As would be expected, more than 85% of the ^{125}I-labeled HDL binding was inhibited by a 300-fold excess of unlabeled HDL. Inclusion of blank egg phosphatidylcholine liposomes (containing no apo A-I or sterols) in the incubation inhibited the binding to some extent, presumably by inhibiting the nonspecific binding of ^{125}I-labeled HDL. However, when apo A-I was incorporated into the liposomes, the binding of ^{125}I-labeled HDL was inhibited even further, lowering it to levels comparable to those obtained in the presence of excess unlabeled HDL. The presence of cholesterol or cholesteryl linoleate in the liposomes did not alter their inhibitory effect on ^{125}I-labeled HDL binding. This experiment shows that the proteoliposomes compete with HDL binding to luteal cell HDL receptors and that cholesterol or cholesteryl linoleate had no role in the inhibition of ^{125}I-labeled HDL binding.

In order to determine directly the effect of apo A-I on the uptake of sterols from egg phosphatidylcholine liposomes, luteal cells were incubated with liposomes containing [^{3}H]cholesterol and [^{3}H]cholesteryl linoleate, and the amount of label taken up was assayed. In the absence of apo A-I, the amount of [^{3}H]cholesterol taken up was about 800 pmol (Fig. 4). Inclusion of apo A-I (50 or 300 μg) in the liposomes increased the uptake mod-

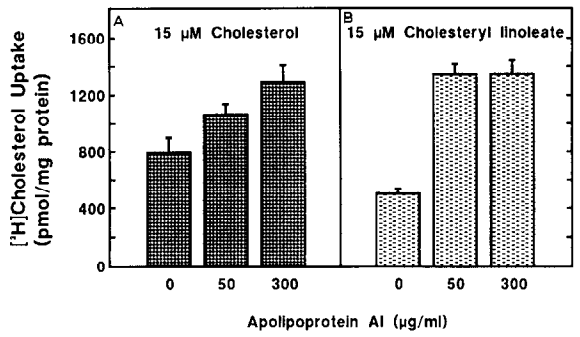

Fig. 4. Effect of apo A-I on the uptake of sterols from egg phosphatidylcholine liposomes containing [^{3}H]cholesterol (A) and [^{3}H]cholesteryl linoleate (B). Cell cultures were incubated with phosphatidylcholine liposomes (300 μg phosphatidylcholine/ml) containing [^{3}H]cholesterol or [^{3}H]cholesteryl linoleate (15 μM final concentration) for 3 h and the amount of cell-associated radioactivity was determined. Values are mean ± S.E. of triplicate determinations.

erately, but significantly. In similar experiments using liposomes containing [^{3}H]cholesteryl linoleate (15 μM final concentration), the amount of label taken up in the absence of apo A-I was much lower when compared to cholesterol liposomes (Fig. 4B). Inclusion of 50 or 300 μg apo A-I in the liposomes resulted in an almost 3-fold increase in the uptake of [^{3}H]cholesteryl linoleate. These experiments show that apo A-I has a pronounced effect on the uptake of cholesteryl linoleate but only a moderate effect on the uptake of free cholesterol.

Steroid synthesis in the presence of egg phosphatidylcholine liposomes containing cholesterol or cholesteryl linoleate showed also a similar effect of apo A-I (Table I). In these experiments, cell cultures were incubated with a constant amount (300 μg/ml) of egg phosphatidylcholine liposomes containing varying amounts (0–100 μM final concentration) of cholesterol or cholesteryl linoleate for 3 h at 37°C, and the amount of progestins released into the medium was assayed. With increasing concentrations of cholesterol in the liposomes, a dose-dependent increase in progestin synthesis was observed. Inclusion of apo A-I along with cholesterol (100 μM) in the liposomes resulted in significantly increased output of progestins (Table I). Thus, it appears that apo A-I further enhances steroid synthesis induced by

TABLE I

PROGESTIN SYNTHESIS IN THE PRESENCE OF EGG PHOSPHATIDYLCHOLINE LIPOSOME CONTAINING CHOLESTEROL OR CHOLESTERYL LINOLEATE WITH AND WITHOUT APO A-I

Luteal cell cultures were incubated with egg phosphatidylcholine liposomes (300 μg phosphatidylcholine/ml) containing cholesterol or cholesteryl linoleate and 100 ng human CG in the absence and presence of apo A-I for 3 h and the amount of progestins released into the medium was assayed. Values are mean ± S.E. of triplicate determinations.

Cholesterol added (μM)	Cholesteryl linoleate added (μM)	Apo A-I added (μg/ml)	Progestin production (pmol)
–	–	–	271 ± 10
10	–	–	432 ± 28
50	–	–	435 ± 35
100	–	–	502 ± 29
100	–	50	630 ± 45
100	–	300	645 ± 48
–	10	–	263 ± 30
–	50	–	244 ± 16
–	100	–	277 ± 9
–	100	50	448 ± 45
–	100	300	515 ± 37

cholesterol liposomes, possibly by promoting receptor-mediated uptake of cholesterol.

Similar experiments using cholesteryl linoleate liposomes (Table I) showed that, in the absence of apo A-I, there was no detectable increase in progestin synthesis. This suggests that cholesteryl esters are not taken up and used for steroidogenesis in the absence of apolipoproteins. However, addition of apo A-I to the cholesteryl linoleate liposomes resulted in a significantly greater progestin production, suggesting the involvement of receptor-mediated uptake of HDL cholesteryl esters for steroid synthesis. Taken together, the above experiments suggest that, in luteal cells, free cholesterol is metabolized by receptor-dependent and receptor-independent processes, whereas esterified cholesterol is metabolized via receptor-mediated processes only.

Discussion

Differential cellular processing of the apoproteins and cholesterol of HDL by adrenal cells,

hepatocytes, fibroblasts and ovarian cells has been reported [18–22]. In these studies, uptake of cholesterol by the cells appeared to be far greater than the amount of HDL apolipoproteins degraded into trichloroacetic acid-soluble products. This discrepancy was explained on the basis of the assumption that HDL may not be internalized. This hypothesis was further strengthened by the observation that in extrahepatic tissues, such as arterial smooth muscle cells and fibroblasts, HDL appears to facilitate cholesterol efflux through a process not necessarily requiring internalization [23–25]. We have reported previously that luteal cells internalize and degrade HDL particles and release the protein components as trichloroacetic acid-soluble and -insoluble products [7]. These studies, which followed the fate of labeled HDL bound to cell surface receptors, also showed that nearly all the HDL-derived cholesterol is retained by the cells. However, the possibility of disparate uptake of free and esterified cholesterol from HDL by luteal cells, which could occur if transfer of free cholesterol between HDL particles and the cell membrane takes place, remained to be examined. In the present study, we have presented experimental evidence for the preferential uptake of free cholesterol from HDL by luteal cells. The amount of HDL-associated free cholesterol taken up by the cells was approximately equal to the amount of esterified cholesterol taken up during the same period. Considering the preponderance of esterified cholesterol over free cholesterol in the HDL particles (5.9 : 1), this represents a 4–6-fold greater uptake of free cholesterol by the luteal cells. The increased uptake of HDL-derived free cholesterol also resulted in its preferential conversion to progestins.

Our results are consistent with the studies of Nestler et al. [26], who reported preferential uptake of free cholesterol from reconstituted HDL by rat granulosa cells. They are also in agreement with the observations of Rothblat et al. [27], who have shown that rat hepatoma cells take up from HDL a greater proportion of free cholesterol compared to esterified cholesterol. The report by Gwynne et al. [28] that HDL containing little or no free cholesterol was less effective than native HDL in supporting adrenal steroidogenesis suggests also the existence of different mechanisms for the processing of free and esterified cholesterol.

The studies using phosphatidylcholine liposomes containing cholesterol or cholesteryl linoleate were performed to understand the mechanisms involved in the disparate uptake of cholesterol and cholesteryl esters from HDL. These experiments suggest that free cholesterol is readily taken up by luteal cells via a receptor-independent process, whereas cholesteryl ester uptake occurs by a receptor-mediated process. Cellular uptake of free and esterified cholesterol from lipoproteins by different mechanisms was suggested by Rothblat [29] as early as 1969. The present studies show that, in luteal cells, receptor-independent uptake of HDL-associated free cholesterol is an important process. Only a fraction (5%) of the HDL-derived cholesterol taken up by the cells during any given time is utilized for steroid synthesis (Figs. 1 and 2). Since free cholesterol uptake alone is sufficient to meet this requirement, it is possible that, in the absence of receptor-mediated uptake, HDL will still be able to sustain steroidogenesis in these cells. This might explain the previous observation that nitrated HDL, which does not bind to receptors, is capable of supporting steroidogenesis [30]. However, our studies also suggest that when receptor binding is not blocked, receptor-mediated uptake of cholesteryl esters and free cholesterol would be the predominant pathway for the uptake of HDL-derived cholesterol.

Acknowledgement

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