Mechanism of the HDL_2 stimulation of progesterone secretion in cultured placental trophoblast

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Abstract Lipoprotein cholesterol (C) supports the high rate of progesterone production by the human placenta as endogenous cholesterol synthesis is low. To study underlying mechanisms whereby lipoproteins, including high density lipoprotein-2 (HDL_2), stimulate progesterone secretion, trophoblast cells were isolated from human term placentas and maintained in primary tissue culture. Lipoproteins were added at several concentrations and medium progesterone secretion was determined. HDL_2 (d 1.063–1.125 g/ml) as well as low density lipoproteins (LDL) (d 1.019–1.063 g/ml) but not HDL_3 (d 1.125–1.21 g/ml) stimulated progesterone secretion in a dose-dependent manner, with HDL_2 cholesterol entering the cell and serving as substrate for progesterone synthesis. Conversely, LDL and HDL_2 produced a significant decrease in [2-^{14}C]acetate incorporation into cell cholesterol. Cholesterol-depleted lipoproteins did not stimulate progesterone secretion. The stimulating effect of LDL was abolished by apolipoprotein modification by cyclohexanedione or reductive methylation and by the addition of anti-LDL receptor antibody or 10 μM chloroquine to the medium. [^{14}C]acetate conversion into cholesterol was accelerated by these procedures. However, HDL_2 stimulation of progesterone secretion and reduction of [^{14}C]acetate incorporation into cholesterol was not blocked by chemical modification of apolipoproteins, anti-LDL receptor antibody, or chloroquine. Treatment of HDL_2 with tetranitromethane or dimethylsuberimidate also did not block the stimulation of progesterone. To determine whether the capacity of HDL_2 to deliver cholesterol to the trophoblast cells was restricted to subfractions differing in apoE content, HDL_2 was chromatographed on heparin-Sepharose and three fractions (A, B, and C) were obtained. Fraction A was poorest in apoE and free cholesterol, fraction B contained the majority of cholesterol, and fraction C was the richest in apoE and free cholesterol. When added to trophoblast cells, fraction A stimulated little progesterone secretion, fraction B stimulated moderately, and fraction C did so greatly. Modification of these subfractions with cyclohexanedione or reductive methylation did not inhibit these effects. In conclusion, HDL_2 stimulated progesterone secretion in human trophoblast cell culture. Contrary to LDL, the HDL effect was not mediated by apolipoproteins or the LDL receptor pathway. The ability of HDL_2 to stimulate progesterone secretion is consistent with the passive transfer of free cholesterol to the cell membrane from a physicochemically specific subfraction of HDL. This mechanism may be an auxiliary source of cholesterol for human steroidogenic cells.—**Lasunción, M. A., B. Bonet, and R. H. Knopp.** Mechanism of the HDL_2 stimulation of progesterone secretion in cultured placental trophoblast. *J. Lipid Res.* 1991. **32:** 1073–1087.

Supplementary key words low density lipoprotein • free cholesterol • apolipoprotein

The human placenta manufactures approximately 400 mg of sex steroids daily (1). Since much of this steroid hormone is derived from plasma cholesterol (2) and little cholesterol is manufactured by the placenta itself (3), the mechanism of transfer of lipoprotein cholesterol to the placenta is of great biological interest. Low density lipoprotein (LDL_2) is a major donor of cholesterol to cells via the LDL receptor (for review, see ref. 4). The LDL pathway has been demonstrated in human trophoblastic cells in primary culture by Winkel et al. (5–7). Binding of ^{125}I-labeled LDL to trophoblastic cells is mediated by high affinity, low capacity specific receptors (7, 8) and LDL stimulates progesterone secretion (5). While specific binding capacity of trophoblastic membrane preparations for ^{125}I-labeled HDL_3 was much greater than for LDL (8), stimulation of progesterone secretion by HDL_3 (d 1.125–1.215 g/ml) was less than by LDL (5). The HDL_2 fraction (d 1.063–1.125 g/ml) was not examined. The conclusion was drawn from these studies that placental biosynthesis of progesterone was principally dependent on cholesterol derived from maternal LDL (5).

More recently, we observed that cultured human trophoblast secretes as much or more progesterone in response to HDL_2 than to LDL_2 when cells are incubated

Abbreviations: LDL, low density lipoproteins; LDL_2, low density lipoproteins d 1.019–1.063 g/ml; HDL, high density lipoproteins; HDL_2, high density lipoproteins d 1.063–1.125 g/ml; HDL_3, high density lipoproteins d 1.125–1.215 g/ml; TNM, tetranitromethane; DMS, dimethylsuberimidate; FBS, fetal bovine serum; LPPS, lipoprotein-poor fetal bovine serum; CE, cholesteryl ester; [^{3}H]CE-HDL_2, HDL_2 labeled with [^{3}H]CE; TLC, thin-layer chromatography.

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at equal concentrations of lipoprotein cholesterol (9, 10). Evidence for HDL_2 delivery of cholesterol to human non-endocrine cells has also been obtained both in the presence and absence of apoE (11, 12). Since apoE competes efficiently with apoB-100 for the LDL receptor (13), the HDL_2 stimulation of progesterone secretion by trophoblast may be due to the presence of apoE in HDL_2, which would then be taken up by cells through the LDL receptor. Alternatively, a distinct apoE receptor that recognizes apoE but not apoB has been described in dog liver (14) and could be present in placenta as well. Another HDL receptor that likely binds apoA-I has been reported in human fibroblasts, arterial smooth muscle cells, endothelial cells, and macrophages (15–17) and is associated with increased binding of HDL_3 when cell cholesterol concentrations are high and with diminished binding when cell cholesterol concentrations are low (18, 19). The chemical nature of this binding site is still controversial (20–22), however a protein that binds apoA-I has been recently purified from human placenta (23). It is not known whether these binding relationships apply to HDL_2.

The purpose of the present work is to determine the mechanism involved in the uptake and utilization of HDL_2 cholesterol by human trophoblast in primary culture. The results demonstrate that HDL_2-stimulated progesterone secretion is associated with apoE-rich subclasses, but that the process is not inhibited as expected by chemical modification of apolipoproteins E and A-I in HDL_2 or by anti-LDL receptor antibody or chloroquine, while the effect of LDL in stimulating progesterone secretion is inhibited as expected by chemical modification of apoB, anti-LDL antibody, and chloroquine. These results suggest a physicochemical specificity for lipoprotein delivery of cholesterol to cells for steroidogenesis that is independent of the B/E receptor and apolipoprotein-mediated binding of HDL to cell membranes.

MATERIALS AND METHODS

Trophoblast isolation and incubation

Two isolation procedures were used. In the first, human term placentas from women undergoing elective caesarian section were used, according to Winkel et al. (5). Fetal cotyledons were dissected and rinsed repeatedly in sterile 0.15 M NaCl. The trophoblastic tissue was teased carefully from the fibrous tissue and the vascular stroma under a sterile UV or laminar flow hood. In a sterile Erlenmeyer flask, the trophoblast was mixed with 50 ml of HAM's F-10 medium (GIBCO, Grand Island, NY) containing 0.25% trypsin (GIBCO) and deoxyribonuclease I (Sigma, St. Louis, MO) and incubated at 37°C in a shaking water bath. After 20 min, the supernatants containing red blood cells and other damaged cells were discarded. The process was repeated twice and these supernatants, now containing largely the trophoblast cells, were poured off over 5 ml of fetal bovine serum (GIBCO) and kept on ice. After filtration through sterile gauze and centrifugation at 800 rpm for 5 min, the cell pellets were combined, suspended in lysing buffer, and incubated on ice for 10 min (5). The cells were collected by centrifugation over 1 ml fetal bovine serum (FBS) and brought to the desired volume with HAM's F-10 medium, pH 7.4, containing 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin, and 2.5 ng/ml fungizone (GIBCO). In later experiments, trophoblast cells were obtained by digesting the minced tissue at 37°C for 60 min in buffered HAM's F-10 medium containing 100 IU/ml collagenase type II (Worthington Biochemical Corp., Freehold, NJ), 0.2 mg/ml hyaluronidase (Sigma), 0.004 mg/ml deoxyribonuclease I (Sigma), and 2% fetal bovine serum (GIBCO). The cell suspension was filtered through sterile gauze, pelleted by low speed centrifugation, and the cells were resuspended in plain HAM's F-10 medium. The trophoblast cells were separated from red cells and macrophages distinguished by immunocytochemical stains (Bonet, B., A. Gown, J. B. Brunzell, and R. H. Knopp, manuscript submitted) by isopycnic centrifugation on linear gradients of 40% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Cells with densities of 1.01–1.02 g/ml were pooled, washed several times to eliminate Percoll, and resuspended in a small volume of HAM's F-10 medium containing 10% fetal bovine serum and the antibiotic mixture as above.

In both isolation procedures, cell number and viability were determined by trypan blue exclusion. Cells were plated in 24-well culture plates (Falcon Primaria, Becton Dickinson, Lincoln Park, NJ) at a rate of 10^6 cells/well. Basal progesterone secretion and also the magnitiude of the response to lipoproteins varied among the experiments, which is related to the cell preparation rather than to lipoproteins.

Lipoprotein isolation and modification

Plasma was obtained from fasted healthy volunteers. Lipoproteins were isolated according to the sequential ultracentrifugation technique (24) in the following ranges: LDL_2 (d 1.019–1.063 g/ml), HDL_2 (d 1.063–1.125 g/ml), and HDL_3 (d 1.125–1.215 g/ml), in a Beckman L5-50 ultracentrifuge. Each fraction was recentrifuged for 24 h at the appropriate density to minimize albumin contamination. The lipoproteins were exhaustively dialyzed against 0.15 M NaCl, 1 mM Na_2EDTA, pH 7.4, for 24 h before use in tissue culture experiments. Cholesterol-depleted lipoproteins were prepared by hexane washing (25).

For incubation, 2-ml aliquots of cell suspensions were plated on Linbro 3.5 × 1 cm multiwell plates (VWR Scientific Co., Media, PA) and maintained at 37°C in a humidified 5% CO_2, 95% air environment. After a 16-h

period to allow the trophoblast cells to adhere to the culture dish, media were changed to HAM's F-10 containing 10% lipoprotein-poor fetal bovine serum (LPPS). Cells were incubated in this medium prior to addition of lipoproteins and the media were changed every 24 h. All media changes and additions were performed under a UV hood or laminar flow hood. Lipoproteins were added at the cholesterol concentration specified in the text for every experiment in the 10% LPPS media containing the antibiotic mixture and fungizone mentioned above. [2-^{14}C]acetate (51 mCi/mmol, New England Nuclear, Boston, MA) was added to media at the final concentration of 20 μM. After a 24-h incubation in the presence of the lipoproteins (or in their absence for the control), the media were collected and the cells were washed twice with 2 ml of 0.15 M NaCl. The cells were collected and extracted according to the procedure of Oram, Albers, and Bierman (26). In later experiments when trophoblasts were incubated with [^{3}H]CE-HDL$_2$, cells were grown in AIM-5 medium (GIBCO).

Cyclohexanedione modification of lipoproteins

The procedure of Mahley et al. (13) was followed. In brief, one volume of lipoprotein solution (ca. 5 mg protein/ml) in 0.15 M NaCl was mixed with two volumes of 0.15 M 1,2 cyclohexanedione (Sigma) in 0.2 M sodium borate buffer (pH 8.1) and incubated for 2 h at 37°C. The samples were then exhaustively dialyzed against buffered 0.15 M NaCl, 1 mM Na$_2$EDTA, pH 7.4. The success in the modification was assessed by agarose gel electrophoresis (Paragon, Beckman, Palo Alto, CA).

Reductive methylation of lipoproteins

Using the procedure of Weisgraber, Innerarity, and Mahley (27), the lipoprotein solutions (ca. 5 mg protein/ml) in 0.15 M NaCl were diluted 1:2.5 with 0.3 M sodium borate buffer, pH 9.0, and placed on an ice bath. For every 5 mg of lipoprotein protein, 1 mg of sodium borohydride was added to the solution at zero time and at 30 min thereafter, with concomitant additions of 1 μl of 37% formaldehyde every 6 min over 60 min. The samples were then chromatographed on Sephadex B-50 to stop the reaction, and the fractions containing the lipoproteins were dialyzed against buffered 0.15 M NaCl, 1 mM Na$_2$EDTA, pH 7.4, and used within the following 5 days.

Tetranitromethane modification of lipoproteins

The method of Chacko (28) and Chacko et al. (29) was followed. Tetranitromethane (TNM) was dissolved in 95% ethanol and added to the HDL solution (2 mg protein/ml) at a rate of 3 μmol TNM/mg HDL protein. The mixture was allowed to stand for 1 h at room temperature and dialyzed in the cold against buffered 0.15 M NaCl, 1 mM Na$_2$EDTA, pH 7.4.

Dimethylsuberimidate modification of lipoproteins

One ml of HDL$_2$ solution (5 mg protein) was mixed with 0.1 ml of 1 M triethanolamine-HCl buffer, pH 9.5, and 5 mg of dimethylsuberimidate (DMS) according to Chacko, Mahlberg, and Johnson (30). After incubation for 2 h at room temperature, the samples were exhaustively dialyzed against buffered 0.15 M NaCl, 1 mM Na$_2$ EDTA, pH 7.4.

Heparin-Sepharose chromatography for HDL$_2$ subfractionation

Heparin-Sepharose CL-6B was purchased from Pharmacia Fine Chemicals AB and packed in a 1.5 × 25 cm glass column. The elution pattern used was that described by Weisgraber and Mahley (31). The heparin-Sepharose was equilibrated at 4°C with 0.05 M NaCl, 0.005 M Tris, 0.025 M MnCl$_2$, pH 7.4. The samples were dissolved in this buffer, loaded onto the column, and after equilibration overnight the same buffer was infused at a rate of 23 ml/h and 3.4-ml fractions were collected. After the first peak was eluted, the buffer was changed to 0.095 M NaCl, 0.005 M Tris, pH 7.4, without manganese for the elution of a second peak. A final increase in the NaCl concentration of the eluent to 0.29 M led to the collection of a third peak. Absorption at 280 nm was monitored and the fractions were appropriately combined and dialyzed against 0.15 M NaCl, 1 mM Na$_2$EDTA, pH 7.4. The recovery of protein applied to the column in six runs was 88 ± 4% and that of cholesterol 85 ± 4%.

Labeling HDL$_2$-CE

HDL$_2$ was labeled with [^{3}H]cholesteryl ester ([^{3}H]CE), adding 50 μCi dissolved in 150 μl of ethanol and 1 ml of a solution containing partially purified lipid transfer protein to the HDL$_2$ solution. This mixture was incubated at 37°C overnight and reisolated by ultracentrifugation at 40,000 rpm for 20 h. With this procedure, the [^{3}H]CE-HDL$_2$ was isolated at the bottom of the tube from the rest of the [^{3}H]CE in the top. About 60% of the total amount of [^{3}H]CE was incorporated into HDL$_2$. In HDL, about 75% of the label was present as cholesteryl ester and 25% as free cholesterol in several experiments, even though the starting labeled cholesterol was 99.7% esterified or more as judged by thin-layer chromatography.

Incubation of trophoblasts with [^{3}H]CE-HDL$_2$

Trophoblasts from human placenta were incubated with AIM-5 medium for 24 h. Medium was then removed and 1 ml of fresh medium containing [^{3}H]CE-HDL$_2$ (sp act 28 cpm/ng cholesterol) was added. Cells were incubated for 24 or 48 h. The medium was removed and the cells were washed 5 times with cold saline. The same process was done in wells with no cells. Lipid extraction

was as described by Hara and Radin (32). The samples were dried under N_2 and redissolved with 125 μl of chloroform. Fifty μl of this solution was used for the thin-layer chromatography (33). Two eluants were used for the TLC, one using petroleum ether–diethyl ether–acetic acid 70:30:1 (v/v/v) that separates cholesteryl esters and a second using the same components at 70:30:2 (v/v/v) that separates free cholesterol and progesterone. The same procedure was done for wells containing no cells and the counts obtained were subtracted from those obtained in wells containing cells.

Analyses

Progesterone was measured in the media by radioimmunoassay using a commercial "coated tube" kit (Diagnostic Products Corp., Los Angeles, CA). Cells were extracted in chloroform–methanol (34) and protein was measured by the method of Lowry et al. (35). The lipid extract was saponified and the nonsaponifiable lipids were extracted in hexane (36), resuspended in isopropanol, and the cholesterol was precipitated with digitonin (37). ^{14}C-radioactivity was determined using a TriCarb 460C Packard liquid scintillation spectrometer. Some culture media were extracted with chloroform–methanol (34) and processed as mentioned for the cells. Assays of cellular free and esterified cholesterol were performed using the enzymatic fluorimetric method of Heider and Boyett (38) before and after saponification.

Total and free cholesterol contents in lipoproteins were measured by commercial enzymatic kits (Sigma). Lipoprotein protein was measured by the method of Lowry et al. (35). SDS electrophoresis was performed on 10% polyacrylamide gels using phosphate buffer as eluant (39) on a Pharmacia Gel Electrophoresis Apparatus GE-4LS. For determining the apparent molecular weight of the lipoproteins, PAA 4/30 gradient gels (Pharmacia Fine Chemicals) were used according to the instructions of the manufacturer. After electrophoresis, the gels were stained with 0.04% Coomassie G-250 in 0.05% perchloric acid. Apolipoprotein immunoassays were performed as described for apoA-I (40), apoA-II (41), apoD (42), and apoE (43).

Results are expressed as mean ± SE. Mean differences between groups were tested by Student's t-paired test and by the multiple range analysis (t-Tukey) using the statistical program, Statgraphics (Statistical Graphics Corp., Princeton, NJ).

RESULTS

Effect of lipoprotein subfractions on progesterone secretion and cell cholesterol content

The addition of increasing amounts of HDL_2 to trophoblast cells in culture produced a dose-dependent increase in the amount of progesterone secreted into the medium (**Fig. 1**) that was clearly detectable at medium cholesterol concentrations of 10 mg/dl. To determine whether lipoprotein-induced increases in progesterone secretion were associated with changes in the amount of trophoblast free and esterified cholesterol, concentrations of these parameters were measured without and with lipoprotein additions in studies in which endogenous cholesterol synthesis was inhibited with compactin. Results are presented in **Table 1** and show that the concentrations of free and esterified cholesterol and the amount of progesterone secreted are higher in the presence of added LDL_2 or HDL_2 but free cholesterol and progesterone are lower in the presence of HDL_3.

To show that HDL_2 can physically provide cholesterol for progesterone synthesis, trophoblasts were incubated for 24 or 48 h with a medium containing [^{3}H]CE-HDL_2. Total lipid extraction of the cells showed an incorporation of ~2% of the total amount of cholesterol added in the medium. On TLC, most of the cellular ^{3}H was present as free cholesterol and less as cholesteryl ester (**Table 2**). At the end of the incubation, the majority of the [^{3}H]cholesterol in the medium (60–80%) remained in the form of esterified cholesterol. Incorporation of [^{3}H]cholesterol into progesterone was consistent and reproducible in the three experiments.

To obtain further evidence that LDL_2 and HDL_2 cholesterol influences progesterone secretion, lipoproteins depleted of cholesterol by hexane washing were added to trophoblast cultures and progesterone secretion was measured. In this experiment all lipoproteins were added at equal protein concentrations. The cholesterol depletion procedure completely removed cholesterol from LDL_2,

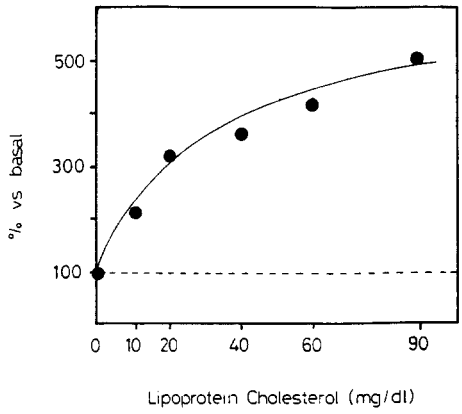


Fig. 1. Effect of HDL_2 on progesterone secretion by trophoblast cells in culture. Trophoblast cells (ca. 10^6 cells/well) were incubated in HAM's F-10 medium containing 10% lipoprotein-poor serum for 48 h and then for 24 h in the same medium supplemented with increasing amounts of HDL_2. Progesterone in the medium was determined and the data were expressed as the percent of the value observed under basal conditions (absence of lipoprotein cholesterol). Values correspond to the mean of two independent experiments in which basal progesterone levels were 2.5 and 11 ng/well, respectively.

TABLE 1. Effect of lipoprotein additions on trophoblast cholesterol content and progesterone secretion

Addition	Cholesterol		Progesterone
	Free	Esterified	
	μg/mg protein		*ng/mg protein*
LPPS + compactin[a]	15 ± 1	4 ± 1	131 ± 40
LPPS + LDL_2	24 ± 2	11	218 ± 88
LPPS + HDL_2	27 ± 11	15 ± 5	262 ± 108
LPPS + HDL_3	9 ± 1	17 ± 2	84 ± 68

Cultures were incubated for 24 h in 10% fetal calf serum. The effects of experimental additions were observed over the next 24 h. Compactin was added at a concentration of 0.5 μM. Values are given as mean ± SE of two or three observations.

[a]Compactin was present in all conditions. Lipoproteins were added at 35 mg/dl cholesterol.

achieved a 45% reduction in HDL_2 cholesterol, but had little effect on HDL_3 (**Table 3**). Unmodified LDL_2 and HDL_2 stimulated progesterone secretion, but less progesterone was secreted in the HDL_2 compared to LDL_2, possibly due to the fact that HDL was added at a lower cholesterol concentration than LDL_2. In the case of cholesterol-depleted LDL_2 and HDL_2 progesterone secretion was reduced to below LPPS control levels. In the presence of HDL_3, progesterone secretion was again reduced below LPPS control. There was no difference between unmodified versus treated HDL_3 on inhibiting progesterone secretion. None of the treatments appreciably altered the cell protein concentrations (Table 3).

Effect of chemical modification of the lipoproteins

To determine whether endocytosis by the LDL receptor or binding to the HDL receptor were involved in the utilization of HDL_2-cholesterol for progesterone secretion, human trophoblast cells were incubated for 24 h in the presence of native and modified LDL, HDL_2, and HDL_3 at two cholesterol concentrations in the media (5 and 20 mg cholesterol/dl). Results illustrated in **Fig. 2** correspond to a representative experiment out of the three performed that gave similar results. Results of progesterone secretion in 24 h are expressed as a percent of basal. As shown in Fig. 2, native LDL and HDL_2 both stimulated progesterone secretion in a dose-dependent manner, and the values reached were similar with both lipoprotein preparations. In contrast, native HDL_3 did not stimulate progesterone secretion at any of the studied lipoprotein concentrations.

Four different procedures were used to determine whether apolipoproteins had a specific role in stimulating progesterone secretion. Cyclohexanedione modification and reductive methylation were used to inhibit lipoprotein interaction with the LDL receptor mediated by either apoB-100 or apoE, and TNM and DMS modifications were used to inhibit binding to the putative HDL receptor. The conditions used for lipoprotein modification with cyclohexanedione and reductive methylation (which modify arginine and lysine, respectively) were those known to inhibit the binding of either apoB-100 or apoE to the fibroblast LDL receptor more than 90% (13, 27). All cyclohexanedione-modified lipoproteins (LDL_2 or HDL_2) showed a faster mobility in agarose electrophoresis than their respective native forms (data not shown). The extent of the reductive methylation modification was not further studied.

As shown in Fig. 2, modification of LDL_2 by cyclohexanedione or reductive methylation reduced progesterone secretion as compared to native LDL_2 such that progesterone secretion was no longer different from that observed under basal conditions. On the other hand, modification of HDL_2 by cyclohexanedione or reductive methylation did not affect progesterone secretion and the values observed were always significantly higher than those in the control wells.

In order to cross-link their apolipoproteins and phospholipids, HDL_2 and HDL_3 were separately modified by TNM treatment (28, 29) and HDL_2 also by DMS (30). The conditions used for these modifications were those

TABLE 2. Uptake of [^{3}H]cholesterol from HDL_2 by trophoblast and incorporation into progesterone

Experiment	Total Lipid Extraction	Free Cholesterol	Cholesteryl Esters	Progesterone
		μg/mg cell protein		
Exp. 1				
24 h	3.68 ± 0.2	2.6 ± 0.2		0.018 ± 0.005
48 h	4.75 ± 1.3	3.5 ± 0.5	0.28 ± 0.02	0.018 ± 0.003
Exp. 2				
24 h	5.04 ± 0.31	2.37 ± 0.5	0.34 ± 0.08	0.020 ± 0.002
48 h	6.80 ± 0.66	2.81 ± 0.28	0.99 ± 0.11	0.023 ± 0.002
Exp. 3				
24 h	3.74 ± 0.41	2.83 ± 0.61	0.52 ± 0.22	0.027 ± 0.01
48 h		3.10 ± 0.38	0.59 ± 0.06	0.024 ± 0.009

Trophoblasts were incubated with 60 μg of [^{3}H]CE-HDL_2 (sp act 29 cpm/mg of cholesterol) for 24 or 48 h. Values are given as mean ± SE of three different wells per experiment.

TABLE 3. Effect of removing cholesterol from LDL_2, HDL_2, and HDL_3 on trophoblast progesterone secretion

Addition	Hexane Washing[a]	Lipoprotein Cholesterol[b]	Medium Progesterone	Cell Protein
		mg/dl	*ng/well*	*μg/well*
LPPS	–	ND	34.8 ± 3.1[c]	198 ± 11
LDL_2	–	22.8	51.8 ± 3.0	186 ± 5
LDL_2	+	ND	25.2 ± 2.9	179 ± 9
HDL_2	–	6.5	43.5 ± 3.5	216 ± 5
HDL_2	+	3.6	24.8 ± 1.7	188 ± 18
HDL_3	–	2.2	18.0 ± 1.3	184 ± 3
HDL_3	+	1.9	21.2 ± 2.9	177 ± 1

Trophoblast cells were incubated for 48 h in LPPS before additions; ND, nondetectable.

[a]Cholesterol was removed from cells by hexane washing (25).

[b]All lipoproteins were incubated at an equal lipoprotein protein concentration of 15 mg/dl (150 μg/ml).

[c]Mean ± SE of three incubations.

known to block the ability of HDL_3 to interact with rat liver or human fibroblast membranes (28–30). TNM-modified HDL_2 and HDL_3 migrated as a broad band and more anodally than native forms in agarose electrophoresis and showed a slower migration in 3–40% polyacrylamide gradient gel electrophoresis, suggesting the presence of particle aggregation in the modified lipoprotein preparations (data not shown). On the other hand, DMS-HDL_2 showed very increased mobility in agarose electrophoresis as compared to native HDL_2 (results not shown).

As also shown in Fig. 2, modification of HDL_2 with TNM or DMS did not affect progesterone secretion as compared to native HDL_2 at either of the cholesterol concentrations in the media, and progesterone secretion observed in the presence of the modified HDL_2 was significantly higher than in the absence of lipoproteins. Likewise, TNM-modified HDL_3 behaved similarly to native HDL_3, the secretion of progesterone in the presence of either modified or native HDL_3 being no different from that observed under basal conditions (Fig. 2).

In another set of experiments, the effect of lipoproteins on the incorporation of [^{14}C]acetate into cholesterol by trophoblast was studied. As shown in **Fig. 3,** native LDL as well as HDL_2 produced a reduction in the appearance of [^{14}C]cholesterol in cells as compared to controls, which is consistent with the stimulation of progesterone secretion seen above. The modification of LDL with either cyclohexanedione or reductive methylation led to a significant increase in [^{14}C]cholesterol appearance as compared to native LDL. In contrast, chemical modification of HDL_2 by these procedures did not produce any change in [^{14}C]acetate incorporation into cell cholesterol, consistent with the divergent effects of chemical modification of LDL versus HDL_2 on progesterone secretion.

Effects of anti-LDL receptor antibody and chloroquine

To confirm that the effect of LDL but not that of HDL_2 was mediated by the uptake of the lipoprotein particle by a receptor-mediated mechanism, human trophoblast cells

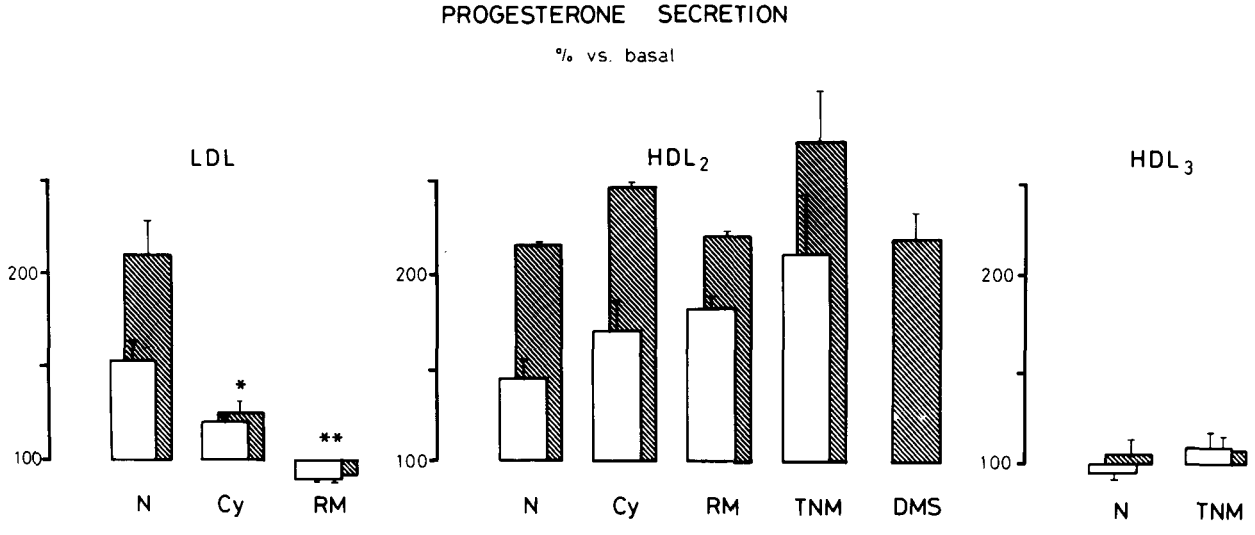
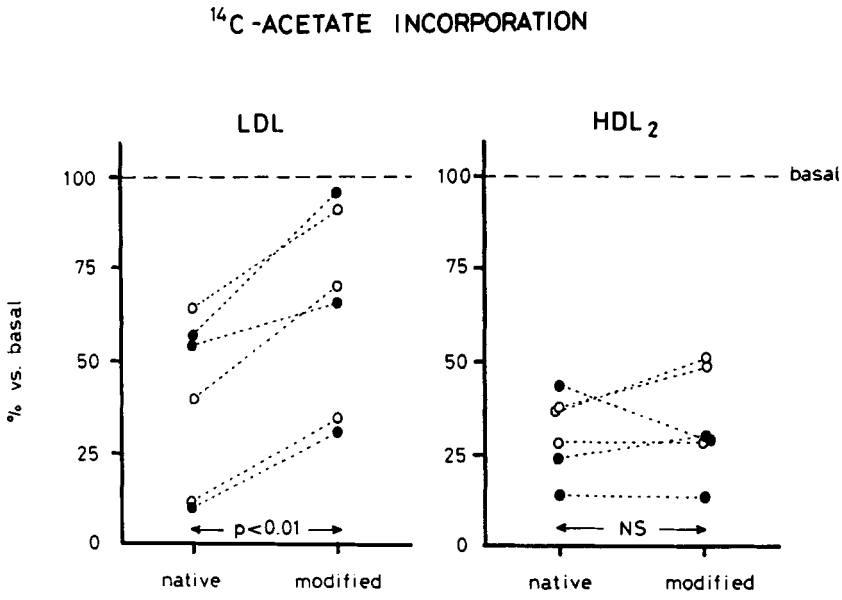


Fig. 2. Effects of cyclohexanedione, reductive methylation, tetranitromethane, and dimethylsuberimidate modification of LDL, HDL_2, and HDL_3 on progesterone secretion by the trophoblast cells in culture. Trophoblast cells (10^6 cells/well) were incubated in HAM's F-10 medium containing 10% lipoprotein-poor serum for 48 h and then for 24 h in the same medium supplemented with 5 (open bars) or 20 (dashed bars) mg/dl of lipoprotein cholesterol. Progesterone in the medium was determined by radioimmunoassay and the data were expressed as the percent of the value observed under basal conditions (absence of lipoprotein cholesterol). Values correspond to the mean ± SE of triplicate incubations from one experiment out of three performed that gave similar results. Progesterone secretion in basal conditions was 12.2 ± 0.4 ng/well. N, native; Cy, cyclohexanedione; RM, reductive methylation; TNM, tetranitromethane; DMS, dimethylsuberimidate. Statistical comparisons between modified and native lipoproteins were performed by the multiple range analysis (*t*-Tukey): * = $P < 0.05$; ** = $P < 0.01$.

Fig. 3. Effects of cyclohexanedione and reductive methylation modification of LDL and HDL_2 on [^{14}C]cholesterol production by trophoblast cells in culture. Trophoblast cells (10^6 cells/well) were incubated in HAMS's F-10 medium containing 10% lipoprotein-poor serum for 48 h and then for 24 h in the same medium supplemented with 20 μM [2-^{14}C]acetate and 40 mg/dl lipoprotein-cholesterol. [^{14}C]cholesterol was determined by digitonin precipitation of an extract of cell lipids. Results are expressed as the percent of the value observed under basal conditions (absence of lipoprotein cholesterol). Each value corresponds to the mean of the triplicate incubations; native, native lipoproteins; modified, modified lipoproteins. Open circles (○) indicate experiments with cyclohexanedione-modified lipoproteins; solid circles (●) indicate experiments with reductive methylation-modified lipoproteins. Statistical comparison between modified and native lipoproteins was done by the *t*-paired test; NS, not significant, $P > 0.05$.



were incubated for 24 h with 20 mg/dl of lipoprotein cholesterol in the presence or the absence of either anti-LDL receptor antibody or chloroquine, and progesterone secretion was determined. Monoclonal anti-LDL receptor antibody was generously provided by Dr. Alan Chait. As in previous experiments, both LDL and HDL_2 stimulated progesterone secretion as compared to basal incubations (absence of lipoprotein-cholesterol) (**Fig. 4**). The addition of anti-LDL receptor antibody or chloroquine to the control wells did not cause any significant effect (data not shown). When these agents were added to wells containing LDL, a significant decrease in progesterone secretion was observed. In fact, in the presence of the anti-LDL receptor antibody, LDL did not significantly stimulate progesterone secretion as compared to basal incubation. In contrast, neither the anti-LDL receptor antibody nor chloroquine modified the effect of HDL_2 stimulating progesterone secretion (Fig. 4).

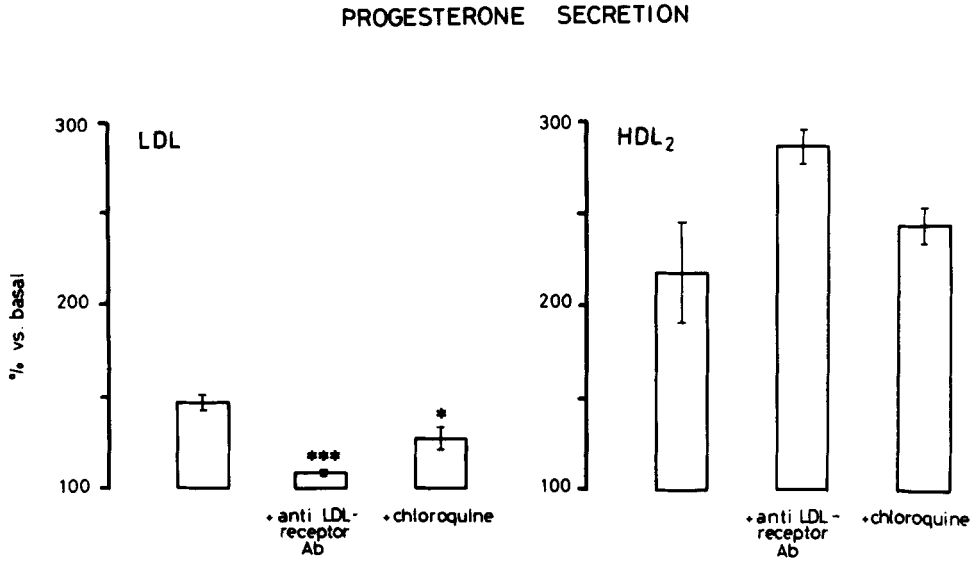


Fig. 4. Effects of anti-LDL receptor antibody and 10 μM chloroquine on progesterone secretion stimulated by LDL and HDL_2. Trophoblast cells (10^6 cells/well) were incubated in HAM's F-10 medium for 48 h and then for 24 h in the same medium supplemented with 20 mg/dl lipoprotein cholesterol and either monoclonal anti-LDL receptor antibody or 10 μM chloroquine, and progesterone in medium was determined. The results are expressed as the percent of the value observed under basal conditions (in the absence of lipoprotein cholesterol), which was 29.2 ± 2.1 ng/well. Values correspond to the mean ± SE of six wells from two independent experiments. Statistical comparisons between the presence versus the absence of anti-LDL receptor antibody or chloroquine were done by the multiple range analysis (*t*-Tukey); * = $P < 0.05$; *** = $P < 0.001$.

Characteristics of HDL_2 separated by heparin-Sepharose affinity chromatography

Although inactivation of apoB-100 and apoE by chemical modification did not inhibit the effect of HDL_2 on steroidogenesis, the apoE content of HDL might still be associated with the HDL_2 effect in some way. To investigate the effect of HDL_2 rich or poor in apoE, HDL_2 was chromatographed on a heparin-Sepharose column in order to separate fractions containing different amounts of apoE (31). HDL_2 from three normolipidemic donors (two females not using birth control pills CL and KP, and one male, MAL) were subfractionated twice each, with high reproducibility. **Fig. 5** illustrates the elution profile for each HDL_2 preparation. Sequential elution with three solutions of increasing NaCl concentration yielded three separate peaks. The first peak was eluted with a 0.05 M NaCl, 0.025 M $MnCl_2$, buffered solution and corresponds to the unbound fraction (fraction A). After increasing the NaCl concentration to 0.095 M, a second sharp peak was collected (fraction B). Finally, the third peak (fraction C) was eluted with a 0.29 M NaCl solution (Fig. 5) (of the total protein added to the column, ranging from 30 to 50 mg, 88 ± 4% was recovered after chromatography). As can be seen in Fig. 5, the elution profiles of the three HDL_2 preparations were similar to one another. In terms of cholesterol, the contributions of the three fractions to the total were 49.8 ± 4.1, 44.3 ± 3.2, and 5.9 ± 1.2%, respectively. In terms of protein, the distribution was 47.6 ± 1.0, 44.7 ± 2.6, and 4.8 ± 0.9 for fractions A, B, and C, respectively.

The cholesterol content, apolipoprotein composition, and cholesterol/protein ratio of the HDL_2 subfractions are shown in **Table 4.** The cholesterol/protein ratio of fractions B and C was higher than that of fraction A. Still, the cholesterol/protein ratio of any HDL_2 fraction was higher than that of HDL_3 (Table 4). The content of free cholesterol versus total cholesterol was the highest for fraction C, intermediate in fraction B, and lowest in fraction A, the differences between them being statistically significant when analyzed by Student's paired *t*-test (Table 4). Free cholesterol content in HDL_3 from the same donors was found to be significantly lower than in total HDL_2 (Table 4).

The apparent molecular weights of the HDL_2 fractions and of HDL_3 were determined by polyacrylamide gradient slab gel electrophoresis. As shown in **Fig. 6** (subject KP), it was possible to estimate a mean molecular mass for each fraction relative to the standards. Fraction A presented a mean molecular mass of 300 kDa, while fraction B was slightly higher, about 330 kDa. Fraction C was comprised of two different populations, a major one remaining at the origin of the electrophoresis and a minor population that migrated as a broad band with a mean molecular mass of approximately 530 kDa, clearly higher than those of fractions A and B (Fig. 6). Fraction A presented a tail that overlapped the HDL_3 band. However, the bulk of fraction A presented a mean molecular mass higher and distinguishable from that of HDL_3, which was 162 kDa (Fig. 6).

The apolipoprotein composition was assessed by SDS polyacrylamide gel electrophoresis and by specific radio-

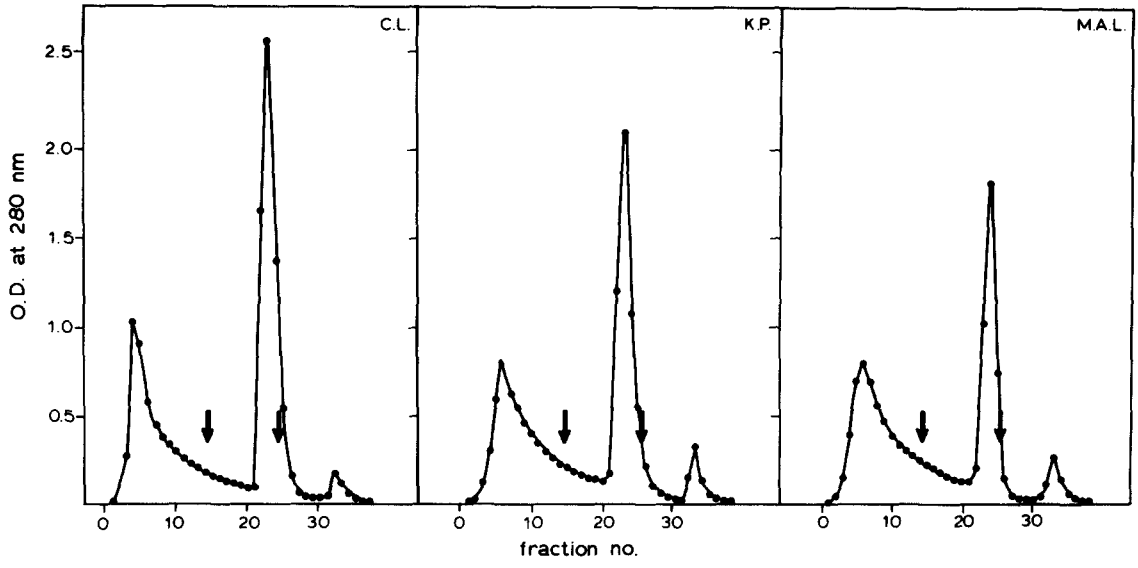


Fig. 5. Elution profiles of HDL_2 by heparin-Sepharose CL-6B affinity chromatography. Approximately 30–50 mg HDL_2 protein in 0.005 M Tris, pH 7.4, containing 0.05 M NaCl and 0.025 M $MnCl_2$ was applied to the column (1.0 × 30 cm). The column was operated at 23 ml/h and 3.4-ml fractions were collected. After the unbound lipoproteins were collected (tubes 1–16, fraction A) the buffer was changed to contain 0.095 M NaCl and no $MnCl_2$ (first arrow). After collecting the second peak (fraction B), the NaCl concentration was increased to 0.29 M (second arrow) to collect the third peak (fraction C). Pooled fractions were dialyzed against buffered 0.15 M NaCl, 1 mM EDTA · Na_2, pH 7.4.

TABLE 4. Compositional studies of HDL subfractions separated by heparin-Sepharose chromatography

Subject	Fraction	A-I/A-II	E/A-I x 10^5	A-I/D	Ch/Prot	% Free Chol
KP	HDL_2	4.20	36.5	40.6	0.35	26.5
	A	2.78	1.2	37.5	0.31	23.3
	B	5.46	47.9	54.2	0.41	28.1
	C		362.2		0.50	48.2
	HDL_3	3.76	10.3	10.8	0.19	16.7
CL	HDL_2	5.13	17.4	50.8	0.37	33.1
	A	3.24	37.4	0.36	0.31	
	B	4.97	8.0	57.7	0.44	36.4
	C		2185.0		0.41	41.3
	HDL_3	5.25		19.0	0.17	15.7
MAL	HDL_2	2.80	18.2	38.5	0.42	29.3
	A	2.00	0.3	35.0	0.41	23.2
	B	3.00	12.5	41.3	0.44	29.7
	C		1720.0		0.44	32.5
	HDL_3	3.00	4.7	16.6	0.20	18.4

Total protein and free and total cholesterol were determined by enzymatic methods, and apoA-I, A-II, E, and D were determined by specific radioimmunoassays (see text for details). Several mass ratios (mg/mg) were calculated for each sample. A, B, and C correspond to the heparin-Sepharose HDL_2 fractions A, B, and C, respectively. The small amounts of fraction C precluded measurement of all apolipoproteins.

immunoassays. **Fig. 7** shows the electrophoretic pattern for KPs lipoproteins and the heparin-Sepharose HDL_2 subfractions (the electrophoretic patterns of lipoproteins of the other two donors were similar to that of KP, data not shown). Every HDL_2 subfraction contained apoA-I, apoE, and other minor polypeptides although in different relative proportions (Fig. 7). Fraction C was the richest in apoE and fraction A the poorest. ApoB was also present in fraction C as in total HDL_3, but not in either fractions A or B (Fig. 6).

To better determine and compare the apolipoprotein composition of HDL_2 subfractions, apolipoproteins A-I, A-II, D, and E were quantitatively measured by radioimmunoassay using specific antibodies. Due to the different albumin content of each lipoprotein preparation, apolipoprotein ratios were calculated (Table 4). As indicated by these ratios, the major apolipoprotein component in fraction A and fraction B as well as in total HDL_2 and HDL_3 was apoA-I. Fraction C of KP revealed apoA-I as the major apolipoprotein, but in CL and MAL, it was apoE. The distinguishing characteristic between the HDL_2 subfractions was their content in apoE as indicated by the ratio apoE/apoA-I. Fraction A was the poorest in apoE, fraction B had an intermediate content, and fraction C was the richest in apoE (Table 4). The content of apoD in total HDL_2 as well as in any HDL_2 subfraction was lower than in HDL_3 as is clearly indicated by the apoA-I/apoD ratios; apoD in fraction C was undetectable (Table 4). It was also found that the relative content of apoA-II in fraction A from each of the subjects was higher than in the respective fraction B, as indicated by the apoA-I/apoA-II ratio (Table 4). ApoA-I/apoA-II ratio in total HDL_2 was found to be similar to that of HDL_3 (Table 4).

Role of HDL_2 subfractions of progesterone secretion by the trophoblast

HDL_2 subfractions separated by heparin-Sepharose column chromatography and total HDL_2 and HDL_3 as well as their counterparts modified by cyclohexanedione or reductive methylation were incubated with human trophoblast cells for 24 h as in previous experiments. As controls, the cells were incubated in 10% LPPS containing medium in the absence of lipoprotein cholesterol. The results of progesterone secretion from three separate experiments are shown in **Table 5.**

In the first experiment (CL in Table 5) the different lipoprotein preparations were added to the cells at the same cholesterol concentration (40 mg/dl). Fraction C was omitted due to its low availability. As in previous experiments, HDL_2 was highly stimulatory whereas HDL_3 did not stimulate any progesterone secretion by the trophoblast. Fraction A hardly stimulated progesterone secretion but fraction B did so significantly (CL in Table 5). Cyclohexanedione modification of the HDL_2 subfractions and total HDL_2 and HDL_3 did not affect the response of the trophoblast to these lipoproteins (CL in Table 5).

In a second experiment (KP in Table 5), HDL_2 subfractions were added at their respective concentrations in 40 mg/dl of total HDL_2 cholesterol, on the hypothesis that their individual effects would add up to equal the total HDL_2 effect. In terms of progesterone secretion, fraction B was still more stimulatory than fraction A, despite being incubated at a slightly lower concentration in the media (KP in Table 5). Fraction C was studied at a very low cholesterol concentration (1.7 mg/dl), nonetheless it clearly stimulated progesterone secretion about fourfold

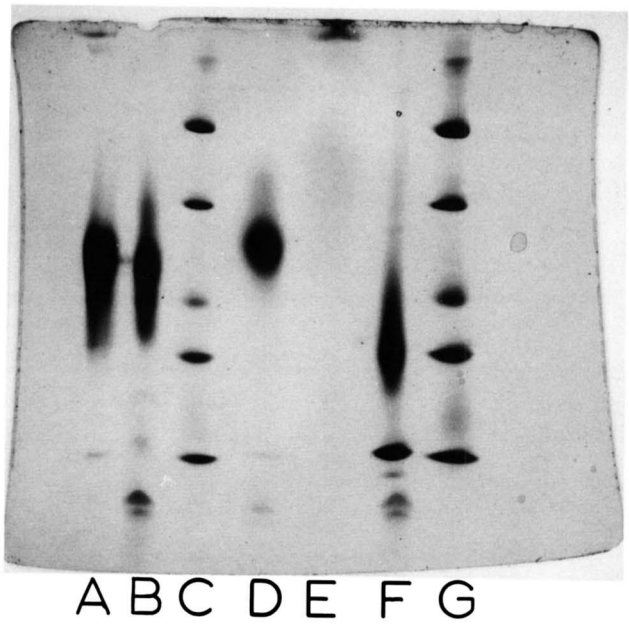


Fig. 6. Polyacrylamide gradient slab gel electrophoresis of heparin-Sepharose HDL_2 fractions, total HDL_2, and HDL_3. Electrophoresis was performed on PAA 4/30 polyacrylamide gradient slabs (Pharmacia Fine Chemicals, Uppsala, Sweden). Approximately 15–20 μg lipoprotein protein was applied to each well. Reference markers correspond to thyroglobulin, ferritin, catalase, lactate dehydrogenase, and bovine albumin. Electrophoresis was performed in Tris 0.09 M, borate 0.08 M, 0.003 M Na_2EDTA, pH 8.35, at 125 V for 16 h. The slab was stained with 0.04% Coomassie blue and destained with 7% acetic acid. Lane A is total HDL_2; lane B is HDL_2, fraction A; lane C contains standards; lane D is HDL_2, fraction B; lane E is HDL_2, fraction C; lane F is total HDL_3; and lane G is another group of standards.

above control (KP in Table 5). In this experiment the effect of reductive methylation of lipoproteins was tested and no significant difference in progesterone secretion between native and modified HDL_2 subfractions was detected (KP in Table 5).

In a third experiment (MAL in Table 5), fractions A and B and HDL_3 were added to the cells at a cholesterol concentration of 15 mg/dl. Fraction C could be only studied at 1 mg/dl, and total HDL_2 was incubated at both concentrations to facilitate comparison. As can be seen in Table 5 (MAL), fraction B strongly stimulated progesterone secretion, the magnitude of the effect approaching that of total HDL_2 at the same lipoprotein concentration in the medium. In contrast, fraction A showed a lesser effect with progesterone secretion similar to that seen with total HDL_3. Fraction C significantly stimulated progesterone secretion despite the low cholesterol concentration of 1 mg/dl. Fraction C was nearly 10-fold more effective than total HDL_2 in promoting progesterone secretion (MAL in Table 5). As in the previous experiments, cyclohexanedione modification of the apolipoproteins did not produce any meaningful change in the ability of total HDL_2 or HDL_2 subfractions to stimulate progesterone secretion nor was there any consistent effect on HDL_3 (Table 5).

DISCUSSION

The production of large amounts of progesterone by the human placenta (1) is sustained by lipoprotein cholesterol (2), as de novo cholesterol synthesis is very limited in this organ (3). While LDL is an established donor of cholesterol to cells (4–7), we previously found that HDL_2 was a potent stimulus for progesterone synthesis and secretion by placental trophoblast cells in culture (9, 10). In the present work we confirm the capacity of HDL_2 to stimulate progesterone secretion. In every experiment, the concentration of progesterone in culture media after a 24-h exposure of trophoblast cells to HDL_2 was higher than in controls (absence of lipoproteins) and of the same order as that observed with LDL_2. In this respect it is noteworthy that the progesterone response to LDL_2 was variable from one experiment to another depending on the trophoblast preparation (especially in those obtained using trypsin for tissue digestion), while HDL_2 was always highly stimulatory. As progesterone in the lipoprotein additions to media was undetectable for any preparation (unpublished data), medium progesterone was necessarily derived from cultured cells. Since [^{14}C]acetate incorporation into cholesterol was depressed in the presence of

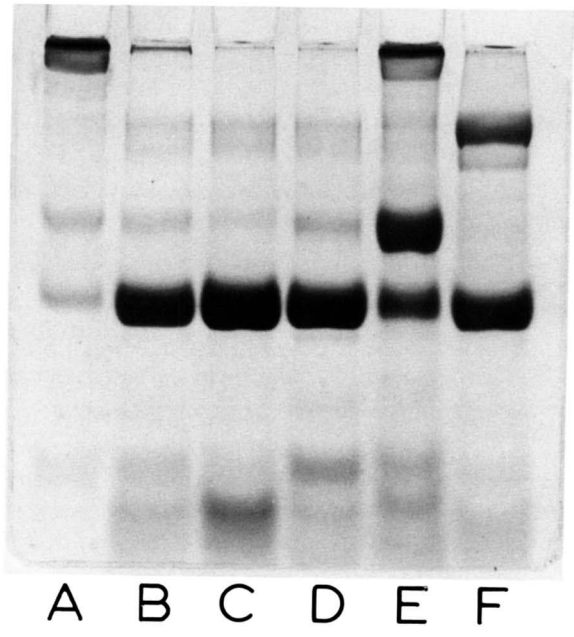


Fig. 7. SDS polyacrylamide slab gel electrophoresis of delipidated lipoproteins on 10% acrylamide gels. Fifty or 150 μg of apolipoprotein (LDL and HDL_2-fraction C) in 0.01 M phosphate buffer (pH 7.4), 0.2% SDS were reduced with 5% mercaptoethanol. Gels were stained with 0.25% Coomassie brilliant blue and destained. Lane A is LDL; lane B is total HDL; lane C is HDL_2, fraction A; lane D is HDL_2, fraction B; lane E is HDL_2, fraction C; and lane F is total HDL_3. From top to bottom the major bands are: apoB, albumin, apoE, apoA-I, and apoA-II plus apoC.

TABLE 5. Effects of HDL subfractions separated by heparin-Sepharose chromatography on trophoblast progesterone secretion

Experiment	Native	Cyclohexanedione-Modified	Reductive Methylated
	ng progesterone/24 h/100 μg cell protein		
1: Lipoproteins from CL			
Basal	37.5 ± 5.1		
HDL_2 (40 mg/dl)	100.7 ± 0.4	113.5 ± 5.8	
HDL_2-A (40 mg/dl)	44.4 ± 1.5	34.5 ± 5.3	
HDL_2-B (40 mg/dl)	86.7 ± 3.8	90.3 ± 10.5	
HDL_3 (40 mg/dl)	26.0 ± 2.8	19.7 ± 2.5	
2: Lipoproteins from KP			
Basal	10.2 ± 7.3		
HDL_2 (40 mg/dl)	103.3 ± 12.5		83.8 ± 7.1
HDL_2-A (20.4 mg/dl)	60.4 ± 10.0		63.4 ± 5.7
HDL_2-B (17.9 mg/dl)	80.0 ± 7.6		87.8 ± 12.2
HDL_2-C (1.7 mg/dl)	37.6 ± 6.2		55.8 ± 12.0
HDL_3 (40 mg/dl)	7.3 ± 6.1		8.0 ± 6.0
3: Lipoproteins from MAL			
Basal	8.6 ± 0.6		
HDL_2 (15 mg/dl)	57.1 ± 3.7	60.4 ± 9.3	
HDL_2 (1 mg/dl)	12.6 ± 3.8	12.5 ± 1.4	
HDL_2-A (15 mg/dl)	24.4 ± 6.5	24.2 ± 4.3	
HDL_2-B (15 mg/dl)	47.9 ± 10.2	38.7 ± 10.7	
HDL_2-C (1 mg/dl)	46.4 ± 7.4	42.8 ± 1.7	
HDL_3 (15 mg/dl)	22.7 ± 7.4	28.1 ± 3.8	

Trophoblast cells (ca. 100 μg cell protein) were incubated in HAM's F-10 medium containing 10% lipoprotein-poor serum for 48 h and then for 24 h in the same medium supplemented with the indicated amount of lipoprotein-cholesterol, and progesterone in medium was determined. Values correspond to the mean ± SE of the triplicate incubations.

HDL_2 as it was for LDL_2 and label derived from HDL-C appeared in cell cholesterol and progesterone, we may assume that HDL_2-C entered the trophoblast cells and was utilized for progesterone synthesis, as is LDL-C (4, 5).

The uptake of HDL_2-cholesterol by cells may involve several receptor-dependent and/or receptor-independent mechanisms. ApoE in HDL_2 may facilitate lipoprotein recognition by the B/E receptor (13). ApoA-I could mediate the interaction with the putative HDL-receptor in several cell types (15–19). Free cholesterol transfer from the HDL particle to the cell could take place in the absence of high affinity HDL binding activity (44–46) when a favorable chemical gradient between lipoprotein and cell membrane exists (46–48). In this connection, hepatic lipase and other phospholipases hydrolyze lipoprotein phospholipids and by increasing the free cholesterol/phospholipid mass ratio in HDL, may enhance the uptake of HDL-free cholesterol by different cell types including hepatocytes (49, 50) and granulosa cells (51).

To better understand the mechanisms of lipoprotein cholesterol uptke by trophoblast cells, we first used the tool of chemical modification of apolipoproteins, and compared effects on LDL_2 and HDL_2. We modified entire lipoproteins by either cyclohexanedione or reductive methylation and, after their addition to the trophoblast cells, found that modified LDL no longer stimulated progesterone secretion as compared to native LDL. This modification did not block the progesterone secretion induced by HDL_2 despite evidence of apolipoprotein modifications by increased mobility in agarose gel. Concomitantly, [^{14}C]acetate incorporation into cell cholesterol, which was significantly depressed in the case of native LDL in comparison to basal incubations, was higher after addition of either arginine- or lysine-modified LDL. No effect of chemical modification of HDL_2 was seen. These results demonstrate that intact apoB-100 is required for LDL to stimulate progesterone secretion and to inhibit cholesterol synthesis from acetate. The HDL_2 stimulation of progesterone secretion is by a different mechanism.

To further explore the role of the LDL receptor, we studied the effect of chloroquine on progesterone secretion. To inhibit lysosomal activity, chloroquine is usually added at a concentration of 100 μM (52) but in preliminary experiments we observed that this high concentration was toxic for the trophoblast in culture (Lasunción, M. A., B. Bonet, and R. H. Knopp, unpublished observations). Therefore, we were obliged to use a lower concentration, 10 μM, in our system. Under these conditions, chloroquine partially inhibited progesterone secretion as a response to LDL without any inherent toxic effect in control experiments, which indicates that lysosomal activity is necessary for LDL processing by the trophoblast cells as in other cells (4). Again, chloroquine had no in-

hibitory effect on HDL_2-mediated stimulation of progesterone secretion.

Direct evidence of the role of the LDL-receptor in mediating the observed effects of LDL versus HDL_2 was obtained by using monoclonal anti-LDL-receptor antibody. This antibody completely suppressed the effect of LDL stimulating progesterone secretion. To our knowledge, this result is the first direct demonstration of the role of the B/E receptor mediating the uptake of LDL-cholesterol for progesterone synthesis in trophoblast and is in accordance with previous results by others describing the stimulatory effect of LDL on progesterone secretion and the LDL binding activity of trophoblast (5, 6). In contrast, anti-LDL receptor antibody did not block the HDL_2 effect.

To determine whether the HDL_2 effect could be ascribed to any particular subfraction, especially to apoE-rich particles, we used heparin-Sepharose column chromatography to separate three different HDL_2 subpopulations. The elution profile of HDL_2 from normolipidemic subjects observed was similar to that described previously by others in human subjects (31, 53) with the exception that in the present work the fraction that eluted at 0.095 M NaCl was the majority, which is probably due to the use of a commercial heparin-Sepharose CL-6B resin instead of a heparin-Sepharose CL-4B prepared from the CNBr-activated form (31). The affinity of the fractions to heparin was correlated with their content of apoE as fraction A (unbound) was the poorest and fraction C the richest in apoE. Fraction C was formed by two different lipoprotein populations as indicated by polyacrylamide gradient gel electrophoresis. Weisgraber and Mahley (31) found that the "bound-II fraction" (equivalent to fraction C herein) was a homogeneous population in terms of apparent molecular mass, with apoB as the sole apolipoprotein, and they concluded that this fraction corresponded to LDL and/or Lp[a] contamination in the HDL_2 density range (31). We also found electrophoretic evidence of apoB, but also detected a smaller amount of apoE and apoA-I in fraction C. Lipid composition of HDL_2 subclasses and HDL_3 was also different. As indicated by the total cholesterol/protein ratio and by the % of free versus total cholesterol, fraction C was richest in free cholesterol and HDL_3 was poorest in free cholesterol.

On incubating these HDL_2 fractions with trophoblast cells, we found that fraction B stimulated more progesterone secretion than fraction A. In fact, except for one preparation, fraction A hardly stimulated progesterone secretion in comparison with HDL_3 or lipoprotein-poor serum (control). When fraction C was studied, we found that its stimulation of progesterone secretion was several times greater than total HDL_2 at the same cholesterol concentration in the media. HDL_2 fraction C was also relatively more effective than fraction B if the different concentrations used are considered. Because of the abundance of apoE in HDL_2 fraction C and the possibility of apoB contamination, we again treated the subfractions with cyclohexanedione or reductive methylation and again found no inhibition of the stimulatory effects of these subfractions on progesterone secretion. These results indicate that the ability of HDL_2 subfractions to deliver cholesterol to the trophoblast cell does not depend upon intact apoE. While apoE-containing HDL_2 could eventually be recognized and taken up by the B/E receptor in their native form, other mechanisms must be involved in the delivery of cholesterol from these lipoproteins to cells in order to explain our observations.

A high affinity binding activity for apoE-free HDL, which recognizes apoA-I, has been reported in many human cell types (15, 16, 18, 19, 54–56). Very recently a protein has also been purified from human placental membrane preparations that binds apoA-I with high affinity (23, 57). This putative HDL receptor has been implicated in the efflux rather than the influx of cholesterol to the cells (18, 19) and it has been suggested that binding of HDL to the cell membrane facilitates sterol translocation between intracellular and plasma membrane pools which allows the transfer of free cholesterol to the HDL particle (17, 44, 55). However, this latter process is known to be independent of HDL binding to the cell (22, 45, 55). To determine whether the effects of HDL_2 and HDL_3 on the trophoblast cells were mediated by the putative HDL receptor, we modified these lipoproteins by treatment with either TNM or DMS. The concentrations of the chemicals and the conditions used were those reported to inhibit HDL binding to cells maximally (28, 30). Confirmation of the extent of the HDL_2 and HDL_3 modification was obtained from nondenaturing polyacrylamide gradient gel electrophoresis and from agarose electrophoresis. When these TNM- or DMS-modified HDL_2 were added to the trophoblast cells, progesterone concentration in the media increased to the same level as with native HDL. HDL_3 were only modified with TNM and these lipoproteins behaved as the native ones, namely, they did not stimulate progesterone secretion as compared to controls even when they were added at a cholesterol concentration in the medium of 20 mg/dl. This inability of HDL_3 to stimulate progesterone secretion was previously observed by Winkel et al. (5), despite the fact that these lipoproteins bind specifically to trophoblast membranes (6). Therefore, present results indicate that HDL effects on trophoblast cells are not mediated by lipoprotein binding to the surface membrane and agree with recent results by others demonstrating that the flux of cholesterol between HDL and fibroblast is independent of apoA-I binding (44, 58) and is not affected by inhibition of HDL binding (45, 46).

There is controversy about the mechanisms for HDL uptake in other steroidogenic cells. In rat corpus luteum cells it was observed that pronase virtually abolished their

capacity to bind ^{125}I-labeled HDL and made them unable to increase progesterone secretion from added HDL (59). However, rat ovarian cell steroidogenesis has been observed by others to be supported by either native or modified HDL, suggesting that HDL can deliver its sterols through low affinity cellular associations (60, 61). A number of recent studies in the rat have reached the conclusion that a large part of the internalized HDL cholesterol occurs via a receptor-independent pathway (12, 58, 62). Our results are in accordance with these observations and suggest that the uptake of free or esterified cholesterol from HDL_2 by the trophoblast cells takes place through a receptor-independent pathway.

In the absence of a demonstrable role for apoE, the factor responsible for the observed differences in the ability of HDL_2 subfractions to support placental steroidogenesis seems to be the free cholesterol content of these lipoproteins. In fact, fraction C had the highest percentage of free cholesterol; fraction B had an intermediate value; and fraction A had the lowest value among the HDL_2 subfractions. If expressed as the free cholesterol/apolipoprotein mass ratio, the differences are even more accentuated. Interestingly, total HDL_3 had a free cholesterol content clearly lower than any HDL_2 fraction. Therefore, it is possible that the free cholesterol content of the HDL particle is related to its effect on progesterone secretion.

The interchange of free cholesterol between lipoproteins and cells has been demonstrated to occur with different cell types and with native as well as apolipoprotein/lipid vesicles (47, 63, 64) and the net movement of this sterol in one or the opposite direction occurs by passive, mass action effects (46). In 1978, Rothblat, Arbogast, and Ray (65) found that HDL enriched in free cholesterol produced sterol accumulation in hepatoma cells and O'Malley, Soltys, and Portman (66) observed that the influx of free cholesterol from HDL to hepatocytes was greater than that from LDL. It was also found that the movement of free cholesterol was not correlated to lipoprotein internalization and degradation (20, 65, 66). Finally, recent studies by others have given evidence that steroidogenic cells preferentially use HDL free cholesterol rather than esterified cholesterol for progesterone synthesis (51, 60, 67) and that free cholesterol is taken up by a receptor-independent pathway (67). Consistent with these observations, our results point to the passive transfer of free cholesterol from a small lipoprotein fraction particularly rich in free cholesterol to the cell membrane, by a process that does not involve the LDL receptor or even the binding of HDL apolipoproteins to the cell. The transfer of a minor part of lipoprotein free cholesterol seems sufficient for supporting progesterone synthesis in the trophoblast in culture as, in an average incubation with total HDL_2, the mass of progesterone secreted is just 1/1000 of the mass of lipoprotein free cholesterol. Therefore, it may be envisioned that in this system there is a chemical gradient of free cholesterol between HDL_2 and the intracellular trophoblast pool that allows net influx into the cell, probably by aqueous diffusion (46, 47). Further research is required to evaluate this hypothesis.

In conclusion, we have confirmed that LDL cholesterol is taken up by trophoblast cells mainly or solely through the B/E receptor, while HDL_2 cholesterol is taken up by a receptor-independent mechanism. The relative physiological importance of LDL versus HDL for the delivery of lipoprotein cholesterol for placental steroidogenesis remains to be elucidated. Nonetheless, in pregnancies carrying fetuses homozygous for familial hypercholesterolemia where the placenta is an obligate homozygote for the defective LDL receptor or in maternal hypobetalipoproteinemia where pregnancies are also viable (68), HDL may provide a reserve or alternative supply of cholesterol to the placenta for progesterone synthesis and transport to the fetus to support fetal growth and development.

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