Cholesterol transport between cells and high density lipoprotein subfractions from obese and lean subjects

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Abstract We studied the pathway of cholesterol efflux from fibroblasts by testing plasma samples from obese and lean subjects. Plasma samples were incubated with [^{3}H]cholesterol-labeled human skin fibroblasts for 1 h to ensure uniform labeling of all of the high density lipoprotein (HDL) subfractions. Supernatants were then transferred to unlabeled cells and the displacement of labeled cholesterol within HDL subfractions by unlabeled cellular cholesterol was analyzed in short-term experiments. Plasma samples of obese subjects were characterized by a lower content of total apolipoprotein A-I (apoA-I) and α_1-HDL and a lower overall capacity to take up labeled cholesterol. In plasma of lean subjects, preβ_2-HDL and α_1-HDL appeared to be the most active particles in the initial uptake of unlabeled cellular cholesterol. By contrast, in plasmas of obese subjects, the preβ_1-HDL appeared to be most active in taking up unlabeled cellular cholesterol and transferring [^{3}H]cholesterol. There were negative correlations between body mass index (BMI) and apoA-I and α_1-HDL concentrations, and with the apparent increments of cellular cholesterol uptake within preβ_2-HDL and α_1-HDL, as well as with the overall capacity to promote cholesterol efflux. By contrast, BMI was positively correlated with the apparent increment in cellular cholesterol within preβ_1-HDL. While cholesterol efflux was correlated with total plasma apoA-1, there were no such correlations with the concentration of any individual HDL subfraction. We conclude that the pattern of cholesterol transfer between fibroblasts and high density lipoprotein particles is influenced by body fatness and may be a factor in the abnormal metabolism of HDL in obesity.—**Sasahara, T., P. Nestel, N. Fidge, and D. Sviridov.** Cholesterol transport between cells and high density lipoprotein subfractions from obese and lean subjects. J. Lipid Res. 1998. **39:** 544–554.

Supplementary key words cholesterol efflux • obesity • high density lipoprotein • preβ-HDL • apolipoprotein A-I • human plasma • lipoproteins • reverse cholesterol transport

It is generally accepted that high density lipoprotein (HDL) plays a central role in reverse cholesterol transport. It is attributed to the ability of HDL to promote cholesterol efflux by removing excess cholesterol from cell membranes, and delivering it to other plasma lipoproteins and to the liver (for review see 1). HDL, however, comprises a heterogeneous group of particles, all containing apolipoprotein A-I (apoA-I), but differing in size, density, lipid and protein content and also in function (2). Non-denaturing two-dimensional electrophoresis of plasma lipoproteins separates HDL subfractions with sufficient resolution to quantitate the participation in cholesterol efflux of the two metabolically interrelated species, preβ-HDL and α-HDL particles (for review see 2, 3). Earlier studies (4, 5) suggested that cellular cholesterol was preferentially taken up by preβ_1-HDL particles and then transferred sequentially to preβ_2-HDL, and preβ_3-HDL and α-HDL particles. Esterification of cholesterol within preβ_3-HDL and α-HDL particles, the cycling of unesterified cholesterol between HDL and LDL, and the eventual transfer of esterified cholesterol from α-HDL to apoB-containing lipoproteins were processes contributing to the redistribution of cellular cholesterol, during cholesterol efflux (4).

Several studies have confirmed the essential features of this pathway, although some inconsistencies have been reported and remained unresolved. For example, the efflux of cholesterol appears to be directly proportional to the size of acceptor particle (6–8) and is influenced by the phospholipid moiety (9), and yet preβ_1-HDL, the smallest HDL particle, with a relatively low phospholipid content (3) is considered one of the most efficient acceptors of cellular cholesterol. It has also been observed that removal of preβ-HDL subfractions from plasma has only a limited effect on its ability to promote cholesterol efflux (10). Further, γ-LpE and LpA-IV lipoproteins appear to have an even greater capacity than preβ_1-HDL for cholesterol efflux (11). Im-

Abbreviations: apoA-I, apolipoprotein A-I; BMI, body mass index; CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein.

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proved two-dimensional electrophoresis allows resolution of several new populations with α-HDL: α_1-HDL, α_2-HDL, and α_3-HDL (12), providing an opportunity to study their role in promoting cholesterol efflux.

In our previous study we compared the distribution of apoA-I among HDL subfractions in the plasmas of lean and obese subjects (13). Obese subjects with several characteristics of metabolic (insulin-resistance) syndrome had lower HDL-cholesterol and apoA-I levels, lower proportions of the larger HDL particles, α_1-HDL and preβ_2-HDL, and higher proportions of the smaller HDL particles, preβ_1-HDL and α_3-HDL. These differences could be partially corrected by changing dietary fatty acids (13) raising the possibility that they may reflect underlying variations in HDL metabolism that affect reverse cholesterol transport. Changes in the distribution of apoA-I among HDL subfractions were observed for subjects with coronary artery disease (14); decreased capacity of plasmas from subjects with non-insulin-dependent diabetes mellitus and coronary artery disease to promote cholesterol efflux has also been reported (15). In the present study we have compared the efficiency of cholesterol efflux in obese and lean subjects, with particular emphasis on the contribution of HDL subfractions to the process. We separated different preβ-HDL and α-HDL subfractions and studied cholesterol transport among them.

MATERIALS AND METHODS

Cells

Human skin fibroblasts were grown in a CO_2-incubator in 75-cm^2 flasks or 6-well tissue clusters (Costar, Cambridge, MA). Cultures were maintained in Dulbecco's modification of Eagle's medium containing 10% fetal calf serum, 20 mm HEPES, 1% non-essential amino acids, 2 mm l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 3.7 mg/ml sodium bicarbonate (all reagents from ICN, Seven Hills, NSW, Australia). Cells were 100% confluent by the beginning of the experiments.

Subjects

Eleven overweight to obese subjects, four men and seven women, were recruited through advertisement. Inclusion criteria were body mass index (BMI) 25–36 kg/m^2, absence of known metabolic disorders other than those resulting from obesity (but excluding diabetes), pharmaco-therapy that might affect lipid metabolism, smoking, and alcohol >20 g/day.

Twelve lean subjects (BMI < 25 kg/m^2), six men and six post-menopausal women, were among the volunteers. Their lipid profiles were normal and they were not receiving medication known to affect lipid metabolism (including hormone replacement therapy).

Relevant vital data for both groups are shown in **Table 1**. The two groups were matched by age and had similar mean plasma total cholesterol and LDL cholesterol concentrations. Obese subjects had significantly higher body weight, BMI, and plasma triglyceride levels and significantly lower plasma HDL cholesterol and apoA-I levels (Table 1).

Informed consent was obtained from each individual prior to blood sampling and the study was approved by the Alfred Hospital Human Ethics Committee.

Blood sampling

Blood samples were taken into heparinized tubes after the subjects had fasted overnight and immediately placed on ice. Plasma samples were obtained by centrifugation at 4°C (2000 *g*, 15 min) and frozen at −80°C. Freezing and thawing of plasma did not affect the distribution of apoA-I-containing lipoprotein subclasses. In some instances apoB-containing lipoproteins were removed from the plasma by precipitation with phosphotungstic acid/$MgCl_2$.

Cholesterol efflux

Cells were labeled with [^{3}H]cholesterol by incubation for 48 h in a CO_2-incubator in 1 ml of complete medium containing [1,2-^{3}H]cholesterol (Amersham, Bucks, UK, specific activity 2.0 TBq/mmol, final activity 3.7 MBq/ml). Cholesterol was added to the medium as an ethanol solution with final ethanol concentration being below 0.1%. Final specific activity of cholesterol in the cells was 0.1 MBq/μg of cholesterol. After labeling, cells were washed 5 times with phosphate-buffered saline and incubated for 1 h with 1 ml of plasma sample

TABLE 1. Clinical characteristics and plasma lipids of lean and obese subjects

	Lean	Obese
Males	6	4
Females	6	7
Age (y)	51.25 ± 10.2	46.20 ± 12.9
Height (cm)	176 ± 8.6	168 ± 8.6[a]
Weight (kg)	70.6 ± 8.8	98.6 ± 16.9[b]
BMI (kg/m^2)	22.8 ± 2.1	35.1 ± 6.6[b]
Total cholesterol (mmol/l)	4.8 ± 0.9	5.1 ± 1.21
Triglycerides (mmol/l)	0.92 ± 0.2	1.88 ± 0.78[b]
HDL-cholesterol (mmol/l)	1.5 ± 0.3	0.94 ± 0.22[b]
ApoA-I (mg/dl)	133.8 ± 12.3	106.7 ± 17.0[b]
LDL-cholesterol (mmol/l)	2.89 ± 0.91	3.34 ± 0.99

All values are mean ± SD.
[a] $P < 0.04$.
[b] $P < 0.001$.

(pulse incubation). After approximately 20 min incubation some transient shrinking of cells was observed which we ascribed to the sharp increase of protein concentration in the medium or to a certain toxicity of 100% plasma. However, cells did not detach from the surface and remained alive according to the Trypan Blue exclusion test, and appeared fully recovered by the end of 1 h incubation. Further, this pulse incubation achieved the desired labeling of cholesterol in HDL subfractions. The actual cholesterol efflux experiments were based on a subsequent 10-min chase incubation during which time no changes in cell morphology were observed. No plasma clotting was observed during pulse or chase incubations. From 6–12% of [^{3}H]cholesterol moved from the cells to plasma as a result of pulse incubation. After the end of pulse incubation, the supernatants were centrifuged for 1 min at 15,000 rpm and transferred to wells with unlabeled cells and incubated for the indicated periods of time (chase incubation). At the indicated periods, 50 μl of the plasma was withdrawn from the well and frozen for further analysis.

Non-denaturing two-dimensional electrophoresis

Non-denaturing two-dimensional electrophoresis, transblotting, and Western blotting were performed as described previously (13, 16) with few modifications. In brief, 20 μl of plasma sample was run on a 0.75% agarose gel (ICN) in 50 mm barbital buffer, pH 8.6 (Bio-Rad, Regents Park, NSW, Australia) at 4°C for 12 h at 50 v. Strips of agarose gel were cut out and laid on the top of vertical slabs of 3–13% polyacrylamide gels (Gradiopore, Pyrmount, NSW, Australia), fixed in position with agarose and the second dimension was performed in 25 mm Tris, 200 mm glycine buffer, pH 8.3, for 1.5 h at 300 v. Lipoproteins were then transferred to the nitrocellulose filters (25 v overnight or 200 v for 2 h in the buffer consists of 25 mm Tris, 200 mm glycine and 10% methanol). Filters were blocked by 2-h incubations in the blocking buffer (10 mm Tris, 1 mm EDTA, 0.15 m NaCl, 3% dry milk). To determine apoA-I distribution among HDL subfractions, Western blotting was performed using rabbit polyclonal anti-apoA-I antibody donkey anti-rabbit IgG and ^{125}I-labeled second antibody (Amersham, specific activity 30–100 TBq/mmol, final activity 8–13 KBq/ml) which were added with the blocking buffer; intermediate washing was carried out with phosphate-buffered saline with 0.1% Tween 20. Nitrocellulose membranes were then exposed to the phosphoimager plates and distribution of apoA-I was evaluated using Bioimager B-1000 (Fuji, Japan). The identity of HDL subfractions was based on the nomenclature suggested by Asztalos et al.(12). The absolute concentration of apoA-I in each fraction was then estimated from the measured apoA-I concentration in the plasma sample and relative distribution of apoA-I among lipoprotein fractions.

The [^{3}H]cholesterol content of HDL subfractions was measured after excision of the fractions from the nitrocellulose filters. This proved to be more precise than using the filters as a template to identify subfractions on the polyacrylamide gel. However, the presence of methanol in the transfer buffer and a detergent in the washing buffer can potentially cause loss of lipids and disruption of lipid composition of various HDL subfractions. To exclude this possibility, recovery of lipoprotein-bound [^{3}H]cholesterol during transblotting was determined in preliminary experiments in which two samples of [^{3}H]cholesterol-labeled plasma (after pulse incubation) were separated by two-dimensional electrophoresis. One sample was transferred to nitrocellulose and HDL subfractions were visualized by Western blotting. This nitrocellulose membrane was then used as a template to identify HDL subfractions in the gel as described by Huang, von Eckardstein, and Assmann (4). By comparing the [^{3}H] cholesterol content in the HDL subfractions excised from the nitrocellulose membrane with corresponding fractions extracted from the polyacrylamide gel, the amounts of [^{3}H]cholesterol in the membrane were, if anything, a little higher than in the gel. For example in one of the experiments, the [^{3}H]cholesterol content of three HDL subfractions responsible for most of cholesterol trafficking, preβ_1-HDL, preβ_2-HDL, and α_1-HDL, were, respectively (membrane versus gel): 66 vs. 55 cpm; 104 vs. 59 cpm; 443 vs. 344 cpm. In no instance did we observe a significant loss of radioactivity from the nitrocellulose membranes. In most experiments, therefore, lipoproteins were transferred to the nitrocellulose membrane. HDL subfractions were then identified by Western blotting, excised, dissolved in methanol, and assayed for radioactivity. Monoclonal anti-apoA-I antibody (AI-4.1 (10)) and goat anti-mouse IgG horseradish peroxidase labeled second antibody (Bio-Rad) were used in these experiments because monoclonal antibody provided more distinct delineation of HDL subfractions.

Analytical methods

Plasma total cholesterol, HDL cholesterol, apoA-I and triglyceride concentrations were determined using the Cobas Bio Scientific Analyzer.

Statistical analysis and model development

The [^{3}H]cholesterol content of various subfractions was expressed in terms of percentage changes relative to that at zero time after reincubation of [^{3}H]cholesterol-labeled HDL (in whole plasma) with fresh unlabeled cells. This was calculated for each time point.

The model assumes that the rates of cholesterol trafficking between HDL subfractions reach steady states after 1 h incubation with labeled cells (pulse incubation). Further incubation of the labeled HDL with new unlabeled cells (chase incubation) assumes no further disturbance in net cholesterol transport (16). Changes in [^{3}H]cholesterol among HDL fractions, therefore, reflect transfer of unlabeled cellular cholesterol to HDL, inducing redistribution of [^{3}H]cholesterol from subfractions with the greatest efficiency for cellular cholesterol to subfractions with a lower efficiency for the initial uptake of cellular cholesterol (discussed later in detail). Therefore the terms "cholesterol efflux" and "capacity to take up cholesterol" are used to describe movements of labeled (pulse) or unlabeled (chase) cellular cholesterol to plasma lipoproteins and not to net transfer of cholesterol mass.

As some HDL fractions lost label through displacement by unlabeled cholesterol, while other fractions accumulated label from the beginning, an "efflux activity" parameter was calculated. This parameter was defined as the area under the curves describing the percentage changes in [^{3}H]cholesterol content from 0 to 5 min relative to the 100% "base line." As the decline in [^{3}H]cholesterol content has been assumed to reflect the ability of the subfraction to match the acquisition of unlabeled cellular cholesterol by a corresponding transfer of label (steady state kinetics), values below 100% have been treated as positive and values above 100% were treated as negative. Correlation coefficients and statistical significance of a correlation were calculated by the Pearson Product Moment Correlation method. Statistical significance of the differences was determined by the Student's two-tailed *t*-test. SigmaStat software (Jandel Scientific) was used for statistical analysis.

RESULTS

Distribution of apoA-I among HDL subfractions

The distribution of apoA-I among HDL subfractions in the plasma samples of obese and lean subjects was similar for the two groups except for a 2.3-fold lower content of α_1-HDL in plasma from obese subjects **(Table 2)**. Obese subjects also had lower plasma apoA-I content (Table 1) and there were statistically significant negative correlations between BMI and total plasma apoA-I ($r = -0.7$, $P < 0.001$) (**Fig. 1A**) and with α_1-HDL ($r = -0.55$, $P < 0.006$)(Fig. 1B). Plasma apoA-I and α_1-HDL concentrations were also positively correlated ($r = 0.57$, $P < 0.005$). Hence, the difference in plasma apoA-I content between obese and lean subjects could be attributed almost entirely to variation in the α_1-HDL subfraction.

TABLE 2. Distribution of apoA-I and [^{3}H]cholesterol in the individual HDL subfractions

HDL Subfraction	Lean Subjects (n = 12)		Obese Subjects (n = 11)	
	ApoA-I	[^{3}H]Cholesterol	ApoA-I	[^{3}H]Cholesterol
	mg/dl	*%*	*mg/dl*	*%*
α_1-HDL	35.2 ± 9.1	36.5 ± 12.3	15.3 ± 4.6[a]	12.5 ± 6.7[a]
α_2-HDL	52.6 ± 9.5	48.9 ± 11.3	48.7 ± 10.6	60.2 ± 16.0
α_3-HDL	22.3 ± 6.3	7.9 ± 3.0	18.5 ± 7.5	23.7 ± 16.1[a]
Preβ_1-HDL	12.8 ± 4.1	1.9 ± 3.2	13.6 ± 5.2	1.0 ± 0.9
Preβ_2-HDL	10.5 ± 2.4	4.8 ± 6.9	9.5 ± 2.8	2.6 ± 1.5
Preβ_3-HDL	0.6 ± 0.3	N.D.	0.7 ± 0.3	N.D.

All values are mean ± SD; N.D., not determined.
[a] $P < 0.001$.

Acquisition of [^{3}H]cholesterol during pulse incubation

A significant difference was observed in the amount of [^{3}H]cholesterol transferred to plasmas of lean and obese subjects during a 1-h pulse incubation:$(12.5 \pm 0.9) \times 10^3$ dpm/μl medium for lean subjects versus $(8.2 \pm 0.8) \times 10^3$ dpm/μl for obese subjects (mean ± SD; $P < 0.001$). This was mainly due to lower plasma apoA-I levels in obese subjects as there was a strong positive correlation between the capacity of plasma to take up cellular cholesterol during pulse incubation and the plasma apoA-I concentration ($r = 0.69$, $P < 0.002$) (**Fig. 2A**). The capacity of plasmas to take up cholesterol was, however, unrelated to the concentration of any individual HDL subfraction during 1 h pulse incubation. It was also unrelated to the activities of the individual HDL subfractions (see Materials and Methods) with the notable exception of preβ_2-HDL: a statistically significant correlation was observed between the capacity of plasmas to take up [^{3}H]cholesterol and preβ_2-HDL activity ($r = 0.61$, $P < 0.01$) (Fig. 2B). The distribution of apoA-I among HDL subfractions was unchanged after pulse incubation.

A difference was also observed when the distribution of [^{3}H]cholesterol among HDL subfractions, after pulse incubation, was compared in lean and obese subjects. The proportion of [^{3}H]cholesterol in the α_1-HDL fraction was 2.9 times less (Table 2), and the amount of [^{3}H]cholesterol recovered in α_1-HDL fraction was 6.7 times less (498 ± 321 versus 3339 ± 1756 dpm; $P < 0.001$) in obese subjects compared to lean subjects. This difference is greater than would be expected as a result of a lower apoA-I content in this fraction and probably indicates that not only quantitative but also qualitative changes occurred in the α_1-HDL subfraction of obese subjects. Proportions of [^{3}H]cholesterol in other α-HDL subfractions were correspondingly increased, this increase reaching statistical significance with the α_3-HDL subfraction. A tendency for a lower [^{3}H]cholesterol content in the preβ-HDL subfractions

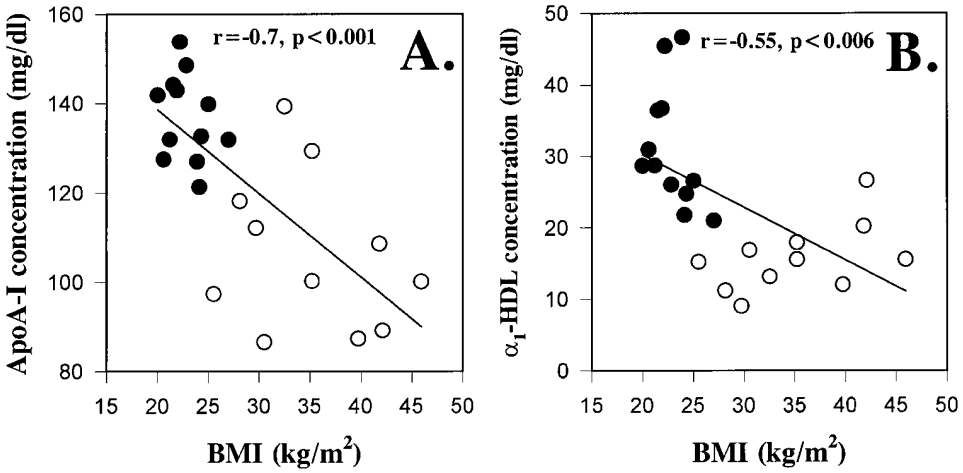

Fig. 1. Relationship between BMI and plasma apoA-I (A) or α_1-HDL (B) concentration. Closed symbols represent lean subjects, open symbols represent obese subjects.

was observed, but this did not reach statistical significance (Table 2).

To ensure that distribution of [^{3}H]cholesterol among HDL subfractions after 1 h pulse incubation reflects a steady state situation, it was compared with the distribution after a 3-h pulse incubation. While the total amount of [^{3}H]cholesteryl in HDL increased 3-fold, the proportions of labeled cholesteryl in preβ_1-HDL, preβ_2-HDL, and α_3-HDL remained constant. There was, however, a redistribution of 15% of [^{3}H]cholesterol from α_2-HDL to α_1-HDL. This redistribution most likely reflects accumulation of cholesteryl esters in α_1-HDL, the particles richest in cholesteryl esters, due to the action of LCAT. Assuming, however, that this redistribution had proceeded linearly during the additional 2 h incubation, its contribution to any redistribution during 10 min chase incubation would be relatively small (probably not exceeding 1.3%); however, possible implication of cholesterol esterification will be discussed in the Discussion section.

Some of the [^{3}H]cholesterol taken up by the lipoproteins during pulse incubations may have been returned to unlabeled cells during chase incubations, mainly through exchange but possibly also via specific mechanisms such as LDL receptors. If so, this was minimal as there was no significant loss of radioactivity from the medium during chase incubation (**Fig. 3**) and the amount of radioactivity recovered in the cells after 1 h

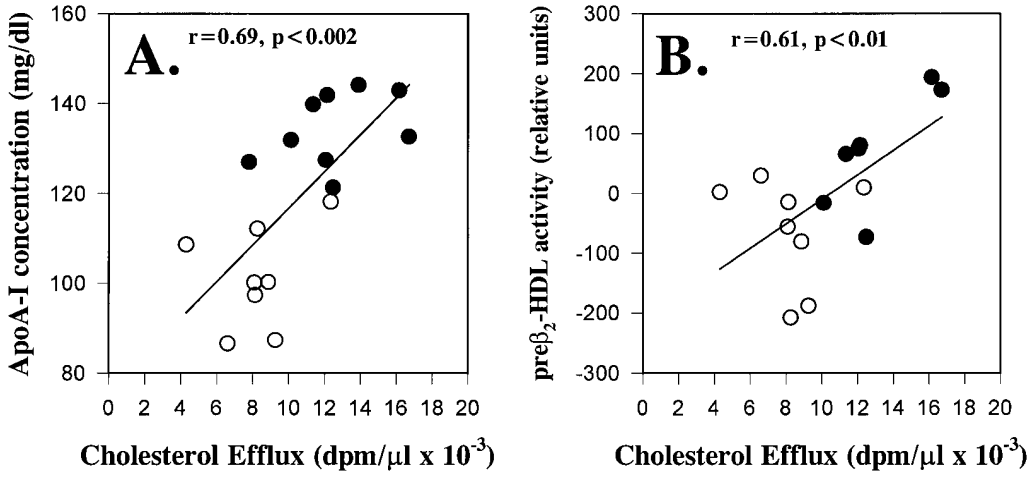

Fig. 2. Relationship between the ability of the plasma sample to promote cholesterol efflux and the plasma apoA-I concentration (A) or preβ_2-HDL activity (B). Closed symbols represent lean subjects, open symbols represent obese subjects.

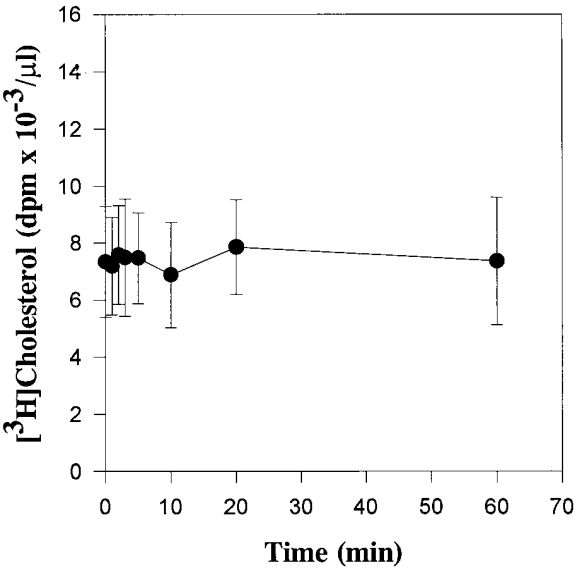

Fig. 3. Time-course of the changes in plasma [^{3}H]cholesterol content during chase incubation. Plasma samples were incubated with [^{3}H]cholesterol-labeled human skin fibroblasts for 1 h at 37°C and then transferred into wells with unlabeled cells. Fifty μl of plasma was removed at the indicated time points of the chase incubation and counted. Mean ± standard error of mean are presented (n = 8).

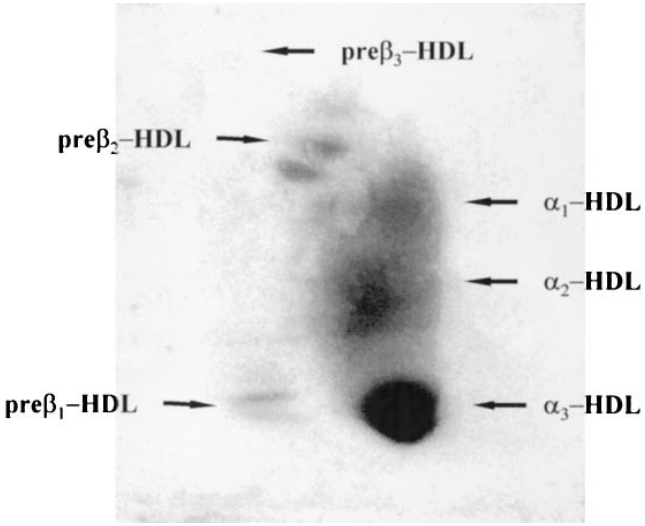

Fig. 4. Western blot of HDL subfractions in the plasma of lean subject. HDL subfractions in the plasma were separated by nondenaturing two-dimensional electrophoresis (see Materials and Methods). After electrophoresis, lipoproteins were transferred to nitrocellulose membranes. ApoA-I-containing lipoproteins were detected using rabbit anti-human apoA-I polyclonal antibody and donkey ^{125}I-labeled anti-rabbit IgG antibody. Note that this figure illustrates resolution of HDL subfractions, but is not quantitatively representative.

chase incubation was less than 2% of that present in the medium. Because our data relate to the events during the first 10 min of the chase, we have discounted reuptake of label.

Cholesterol trafficking in the plasma of lean subjects

Redistribution of [^{3}H]cholesterol among HDL subfractions during the chase incubation was analyzed by two-dimensional electrophoresis; using the nomenclature suggested by Asztalos et al.(12) HDL subfractions are termed preβ$_1$-HDL, preβ$_2$-HDL, preβ$_3$-HDL, α$_1$-HDL, α$_2$-HDL, and α$_3$-HDL (**Fig. 4**). Distribution of apoA-I and [^{3}H]cholesterol among these subfractions before the beginning of the chase incubation is presented in Table 2.

Redistribution of [^{3}H]cholesterol among HDL subfractions was analyzed in time-course experiments. The amount of [^{3}H]cholesterol in each subfraction is expressed as the percentage change and was normalized to the amount of [^{3}H]cholesterol present in this subfraction at the beginning of chase incubations. Most of the [^{3}H]cholesterol was redistributed among HDL subfractions within the first 5–10 min incubation with little further change thereafter.

The amount of [^{3}H]cholesterol in the preβ$_2$-HDL fraction of the plasma from lean subjects decreased rapidly on average, suggesting that this was the most active fraction in taking up unlabeled cholesterol and transferring [^{3}H]cholesterol to other lipoproteins (**Fig. 5A**). On the other hand, the amount of [^{3}H]cholesterol in the preβ$_1$-HDL fraction increased on average from the beginning (Fig. 5A), suggesting that this fraction accepted [^{3}H]cholesterol from other lipoproteins, while not excluding some uptake of unlabeled cholesterol from cells. Among the α-HDL fractions from lean subjects, the amount of [^{3}H]cholesterol in the α$_1$-HDL fraction initially decreased while that in α$_2$-HDL and α$_3$-HDL increased (**Fig. 6A**). After 3 minutes, label in the α$_1$-HDL fraction started to increase also. This is consistent with more unlabeled cholesterol being initially taken up by α$_1$-HDL, the largest HDL particle, and corresponding transfer of labeled cholesterol to α$_2$-HDL and α$_3$-HDL. The data suggest that both preβ$_2$-HDL and α$_1$-HDL participated most actively in the initial uptake of cellular cholesterol in lean subjects.

The amounts of apoA-I and [^{3}H]cholesterol in preβ$_3$-HDL were below 0.01% and 0.5% of the total, respectively, and no consistent pattern of redistribution of [^{3}H]cholesterol to or from these particles was observed.

Cholesterol trafficking in the plasma of obese subjects

There was no significant change in the [^{3}H]cholesterol of preβ$_2$-HDL, while that in preβ$_1$-HDL declined in the plasma of obese subjects (Fig. 5B). Significant differ-

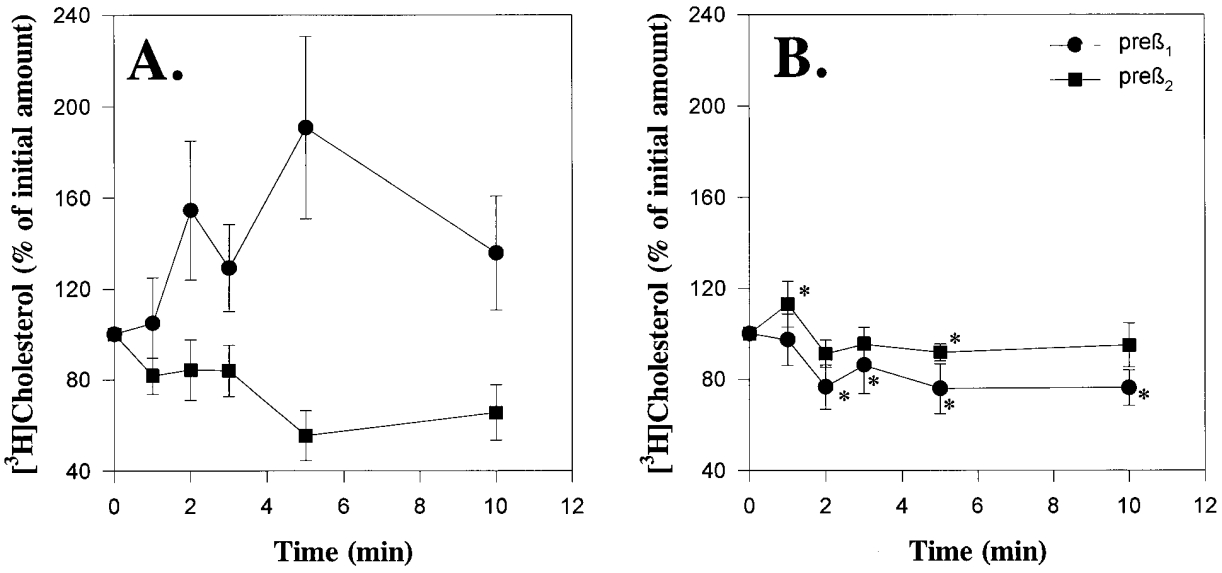

Fig. 5. Time-course of the redistribution of [^{3}H]cholesterol among preβ-HDL subfractions in plasmas of lean (A) or obese (B) subjects during chase incubations. Plasma samples were incubated with [^{3}H]cholesterol-labeled human skin fibroblasts for 1 h at 37°C and then transferred into wells with unlabeled cells and incubated for the indicated times at 37°C. Fifty μl plasma was removed at the indicated time points of the chase incubation. Preβ-HDL subfractions were separated by non-denaturing two-dimensional electrophoresis, transferred to the nitrocellulose membranes, and developed using monoclonal anti-human apoA-I antibody and HRP labeled goat anti-mouse IgG antibody. Areas corresponding to preβ_1-HDL and preβ_2-HDL subfractions were excised, dissolved in methanol, and counted. Radioactivity in each subfraction before the beginning of the chase incubation (time point 0) was taken as 100%, and values for the following time points were calculated relative to this initial value (see Materials and Methods for details). Means ± standard error of mean are presented. *$P < 0.05$ (versus corresponding values for lean subjects at A).

ence in the amounts of [^{3}H]cholesterol in preβ_1-HDL and preβ_2-HDL between lean and obese subjects was observed at most time points. Plasmas of obese subjects were characterized by 2-fold lower content of α_1-HDL, and the [^{3}H]cholesterol content of this fraction changed very little during the first 3 min of chase incubation, but declined thereafter (Fig. 6B). Both α_2-HDL and α_3-HDL fractions accumulateed [^{3}H]cholesterol during the chase incubation in a manner similar to that in lean subjects (Fig. 6B). The only significant change after 5 min of chase incubation was a gradual decline in the amount of [^{3}H]cholesterol in α_1-HDL of both obese and lean subjects.

Relationship between efflux activities of HDL subfractions and obesity

In order to estimate the relative activity of each HDL subfraction to stimulate cholesterol efflux, a value of activity was calculated as described in Materials and Methods. Activity of preβ_1-HDL correlated positively with BMI ($r = 0.46$, $P < 0.05$)(**Fig. 7A**) which is consistent with the finding of an initial decline in preβ_1-HDL [^{3}H]cholesterol in obese subjects (Fig. 5). On the other hand, BMI correlated negatively with the activities of preβ_2-HDL ($r = -0.47$, $P < 0.05$) (Fig. 7B) and α_1-HDL ($r = -0.46$, $P < 0.05$)(Fig. 7C) which is also consistent with these HDL subfractions from lean subjects being more active in exchanging cholesterol. Changes in the activities of these fractions were interdependent: there was a positive correlation between activities of preβ_2-HDL and α_1-HDL ($r = -0.58$, $P < 0.02$) and a negative correlation between activities of preβ_2-HDL and preβ_1-HDL ($r = -0.5$, $P < 0.04$). Surprisingly, there was also a negative correlation between α_1-HDL and α_2-HDL activities ($r = -0.6$, $P < 0.01$) (**Fig. 8**). This may suggest that α_1-HDL and α_2-HDL are either competing for cellular cholesterol, a high activity of one decreasing the availability of cellular cholesterol to the others, or that they are in a precursor–product relationship. Interestingly, there was no correlation between the activity of each fraction and its concentration in plasma. There were no correlations between BMI and activities of α_2-HDL or α_3-HDL nor between plasma triglyceride level and the activity of any HDL fraction.

Cholesterol efflux to apoB-free plasma

Because some cholesterol that appears in different HDL subfractions may originate from LDL rather than cells, we compared trafficking of cholesterol among

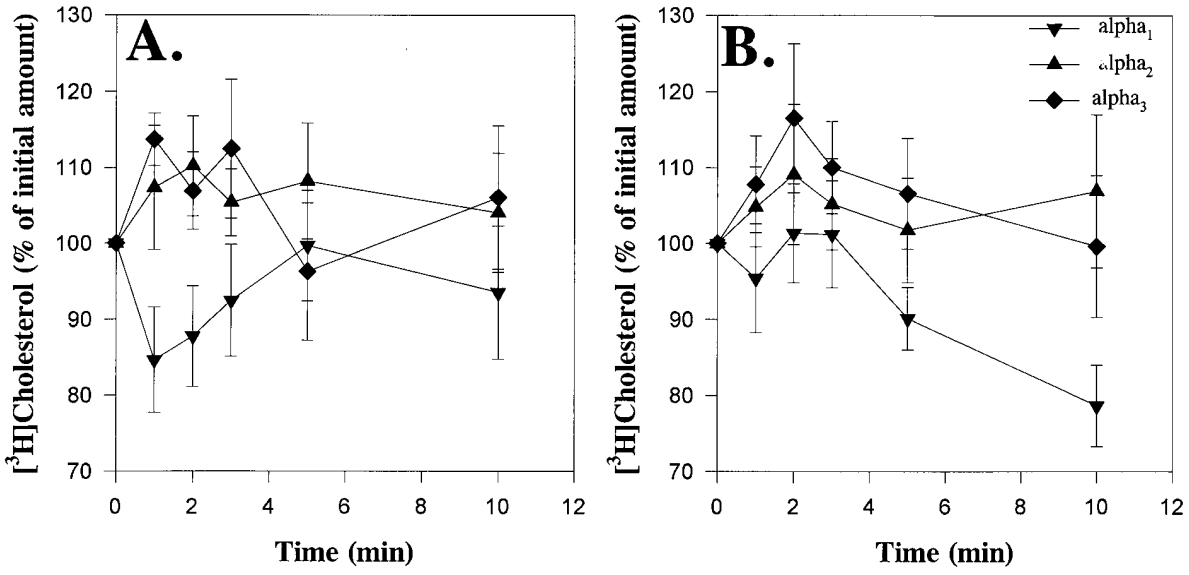

Fig. 6. Time-course of the redistribution of [^{3}H]cholesterol among α-HDL subfractions in plasmas of lean (A) or obese (B) subjects during chase incubations. Plasma samples were incubated with [^{3}H]cholesterol-labeled human skin fibroblasts for 1 h at 37°C and then transferred into wells with unlabeled cells and incubated for the indicated times at 37°C. Fifty μl of plasma was removed at the indicated time points of the chase incubation. α-HDL subfractions were separated by non-denaturing two-dimensional electrophoresis, transferred to nitrocellulose membranes, and developed using monoclonal anti-human apoA-I antibody and HRP labeled goat anti-mouse IgG antibody. Areas corresponding to α_1-HDL, α_2-HDL, and α_3-HDL subfractions were excised, dissolved in methanol, and counted. Radioactivity in each subfraction before the beginning of the chase incubation (time point 0) was taken as 100%, and values for the following time points were calculated relative to this initial value (see Materials and Methods for details). Means ± standard error of mean are presented.

HDL subfractions in whole and apoB-free plasma. The relative distribution of neither apoA-I nor [^{3}H]cholesterol after pulse incubation among different HDL subfractions was affected by precipitation of the apoB-containing lipoproteins. To investigate whether the presence of apoB-containing lipoproteins might have affected trafficking of cholesterol between HDL subfractions during cholesterol efflux, whole and apoB-free plasmas from one lean and one obese subject were compared. Relative changes in the [^{3}H]cholesterol content of preβ-HDLs and α-HDLs were consistent with the changes described for pooled data and there was no difference between incubations with or without apoB-containing lipoproteins (not shown).

DISCUSSION

In this study we investigated the pathway of cholesterol efflux from cultured human skin fibroblasts to hu-

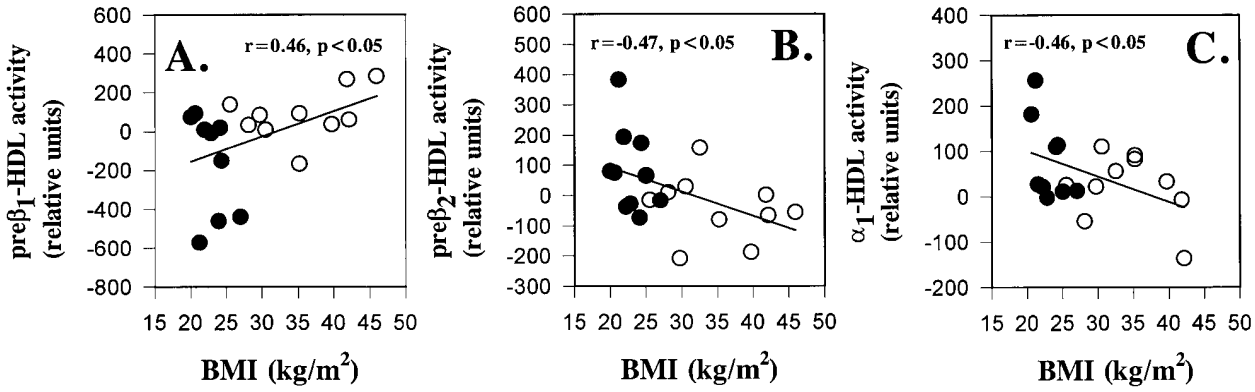

Fig. 7. Relationship between BMI and activities of preβ$_1$-HDL (A), preβ$_2$-HDL (B), and α$_1$-HDL (C). Closed symbols represent lean subjects, open symbols represent obese subjects.

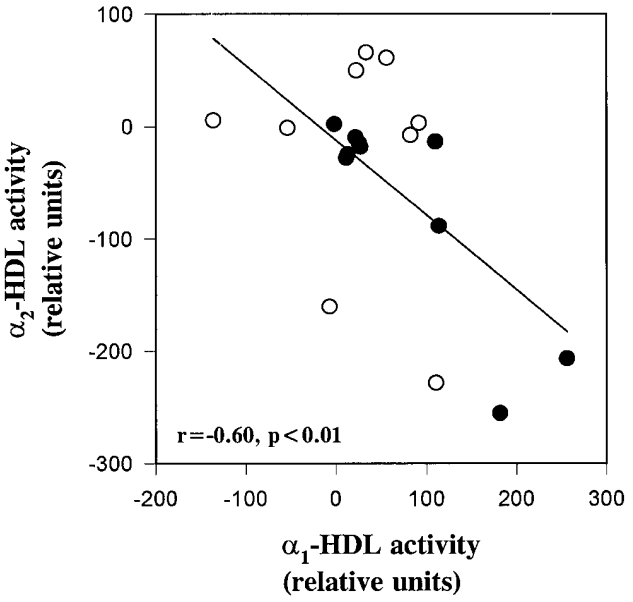

Fig. 8. Relationship between activities of α_1-HDL and α_2-HDL. Closed symbols represent lean subjects, open symbols represent obese subjects.

man serum. Compared with other studies, *i*) we used an improved technique of non-denaturing two-dimensional electrophoresis, which distinguished between different α-HDL subpopulations, *ii*) compared cholesterol efflux in plasmas of lean and obese subjects, and *iii*) used an experimental design based on steady state conditions while quantitating cholesterol trafficking among different HDL subfractions and cells.

Several studies have demonstrated that during the efflux of cellular cholesterol, the latter is preferentially taken up by preβ_1-HDL particles and then unidirectionally transferred to preβ_2-HDL, preβ_3-HDL and finally to α-HDL particles (4, 5, 11, 17, 18). The experimental design used in such studies included a short (1–5 min) pulse incubation with labeled cells, followed by a chase incubation with unlabeled cells. "Pulse wave" of labeled cholesterol moving from one HDL subfraction to another was monitored over time (4, 5, 11, 17, 18), and demonstrated precursor–product relationships between different HDL subpopulations. However, we have shown previously that when cells are incubated with human serum, it takes about 1 h to achieve an equilibrium between cholesterol in cells and in serum. After that time there is no further net transfer of cholesterol between cells and serum, and the rates of cholesterol exchange become constant (16). Prior to equilibration, cholesterol is preferentially taken up by the particle with the highest capacity to bind cholesterol, characteristically having a low cholesterol/phospholipid ratio. As efflux progresses, however, these particles become enriched with cholesterol and net uptake of cellular cholesterol declines, presumably limited by the rate of production of new "fresh" particles. Accordingly, preβ_1-HDL particles, having a relatively low cholesterol/phospholipid ratio (3), may be the most active net acceptors of cholesterol for the first 1–5 min, but as the cholesterol concentration in the particles rises, the flux of cholesterol through these particles may decline. We have, in fact, shown that the amount of labeled (cellular) cholesterol in preβ_1-HDL particles declined during an extended pulse incubation (16).

To overcome this problem we extended the pulse incubation to 1 h to achieve a steady state of cholesterol trafficking between cells and the plasma. Events during the subsequent chase are schematically presented in **Fig. 9**. After transfer of plasma with labeled HDL to fresh unlabeled cells, unlabeled cellular cholesterol (Fig. 9, open arrows) displaced [^{3}H]cholesterol in those HDL subfractions that are characterized by the highest association–dissociation rates for cellular cholesterol. In a steady state situation, these particles will be most active in taking up cellular cholesterol, transferring equal amounts of labeled cholesterol (Fig. 9, closed arrows) to another acceptor. It was postulated that the transfer of cholesterol through preβ-HDL particles would be largely, through not necessarily entirely, unidirectional, as suggested by Barrans et al.(3). After reaching α-HDL, cholesterol would shuttle between different α-HDL subfractions and also between HDL, LDL (not shown), and cells (Fig. 9, dashed arrows). Subsequently, it would be esterified by LCAT and partially transferred to LDL through the action of CETP. According to this model, particles with high association–dissociation rate will be more efficient in transferring their labeled cholesterol to other lipoproteins and therefore will lose labeled cholesterol and accumulate unlabeled cholesterol, while the particles with lower association–dissociation rates as well as mature particles would be expected to accumulate labeled cholesterol.

We found that in plasmas of lean subjects preβ_2-HDL was the most active preβ-HDL subfraction involved in cellular cholesterol uptake. During chase incubations the amounts of labeled cholesterol in this fraction decreased rapidly, presumably as a consequence of early uptake of unlabeled cholesterol from cells. This was unrelated to the presence or absence of apoB-containing lipoproteins, excluding LDL as a source of unlabeled cholesterol. Preβ_2-HDL was the only HDL subfraction with activity that correlated significantly with cholesterol efflux. Preβ_2-HDL particles have the lowest unesterified cholesterol/phospholipid ratio among all HDL subfractions (3) and they are relatively large particles. These properties are likely to enhance their activity in cellular cholesterol uptake and would be consistent with the present findings.

On the other hand, in lean subjects, preβ_1-HDL was

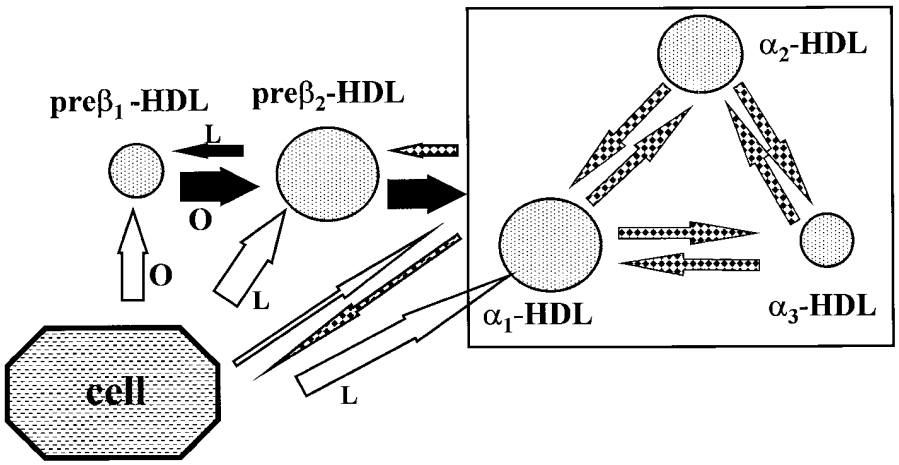

Fig. 9. Schematic representation of pathway of cholesterol trafficking between cells and HDL subfractions. Open arrows represent unlabeled cellular cholesterol, closed arrows represent labeled cholesterol, and dashed arrows represent equilibrated cholesterol pool; a box represents pool of α-HDL, where unesterified cholesterol is equilibrated by free exchange among the particles. In lean subjects (arrows "L") unlabeled cellular cholesterol is taken up preferentially by preβ$_2$-HDL displacing labeled cholesterol from these particles to preβ$_1$-HDL and to the α-HDL pool. α-HDL particles also exchange cholesterol with cells with α$_1$-HDL of lean subjects being the most efficient in taking up cellular cholesterol. In obese subjects (arrows "O") preβ$_1$-HDL is the preferred acceptor of cellular cholesterol, displacing labeled cholesterol from these particles to other HDL subfractions.

not as active in the initial uptake of cellular cholesterol, and received more [^{3}H]cholesterol than unlabeled cellular cholesterol, probably from preβ$_2$-HDL, although other lipoproteins, including γ-LpE or LpA-IV, which have also been shown to take up cellular cholesterol during pulse incubations (11, 17), might also have been donors. Preβ$_1$-HDL is a small particle, which might lessen its activity as an acceptor of cellular cholesterol, but on the other hand, its relatively low cholesterol/phospholipid ratio should enhance its ability to accept cholesterol from other lipoproteins. Alternatively, the increase in the amount of [^{3}H]cholesterol in the preβ$_1$-HDL fraction during chase incubations may have resulted from the formation of new preβ$_1$-HDL particles from heavily labeled α-HDL. Consistent with the last possibility, there was no increase of [^{3}H]cholesterol content in preβ$_1$-HDL in the plasmas of obese subjects that have a relative deficiency in α$_1$-HDL. While our findings appear to be inconsistent with the earlier findings of Castro and Fielding (5) and Huang et al. (4), as well as our own (16), this most likely reflects the differences in the experimental design. Whereas preβ$_1$-HDL that had not been in recent contact with cells becomes an active acceptor of cellular cholesterol, this capacity becomes reduced after a lengthy preincubation, as in the present study. Continuous participation of preβ$_1$-HDL in cholesterol efflux would be limited by the rate of their production during metabolism of α-HDL.

When transport of cholesterol through the α-HDL subfractions was analyzed, α$_1$-HDL was found to be most active in taking up unlabeled cholesterol. This agrees with the findings of Davidson et al.(7), Agnani and Marcel (6), and Zhao, Sparks and Marcel (8) who showed that the largest particles are the most effective acceptors of cellular cholesterol. α$_2$-HDL and α$_3$-HDL subfractions, on the other hand, accumulated [^{3}H]cholesterol, the source of which may have been α$_1$-HDL. α$_3$-HDL consists mainly of HDL$_3$, the smallest α-HDL particle, which is a preferred substrate for LCAT (19). It can be assumed that labeled cholesterol in the α$_3$-HDL subfraction is rapidly esterified, and may reappear again in the α$_1$-HDL subfraction. The observed transfer of cholesterol between α-HDL subfractions may, therefore, be a balance between unesterified cholesterol shuttling between the particles and transformation of one particle into another through esterification of cholesterol.

The major differences in cholesterol efflux between plasma from obese and lean subjects were the higher activity of preβ$_1$-HDL, the lower activity of preβ$_2$-HDL, lower activity and concentration of α$_1$-HDL, and overall lower ability to promote cholesterol efflux in the obese. Thus, in lean subjects, the preferred acceptor of unlabeled cellular cholesterol is preβ$_2$-HDL, with subsequent transfer of labeled cholesterol to preβ$_1$-HDL and the α-HDL pool; α$_1$-HDL is also a preferred acceptor (Fig. 9, arrows "L"). In obese subjects preβ$_1$-HDL was the preferred acceptor of cellular cholesterol, followed

by transfer of labeled cholesterol to the α-HDL pool possibly via preβ$_2$-HDL (Fig. 9, arrows "O"). Three possible explanations for these changes in the obese subjects are offered. Because HDL catabolism is increased in obesity (20), this is likely to apply mostly to α$_1$-HDL, lowering its concentration and resulting in formation of preβ$_1$-HDL. Such preβ$_1$-HDL particles may have an altered lipid profile which may increase their activity in taking up cellular cholesterol. The second possibility is that conversion of preβ$_1$-HDL to preβ$_2$-HDL (which is most likely to be a fusion, because of lack of intermediate-sized particles) may be impaired with the formation of less active preβ$_2$-HDL. A third possibility is that the HDL particles in obese subjects are qualitatively as well as quantitatively different from those in lean subjects because of events within peripheral cells in the obese. Intracellular metabolic changes that alter cholesterol efflux might be expected to change the nature of the subsequent process of cholesterol transport among HDL subpopulations. The different patterns in preβ-HDL activities in lean and obese subjects occurred without changes in their concentrations (measured as apoA-I). Lack of correlation between cholesterol efflux and the concentration of any single HDL subfraction, most notably of α$_1$-HDL (which determined the difference in the HDL concentration between plasmas of obese and lean subjects) suggests that the qualitative rather than quantitative changes in the HDL subfractions were mainly responsible for the differences in cholesterol efflux.

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