Evidence for non-equilibrating pools of apolipoprotein C-III in plasma lipoproteins

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Abstract Using immunoaffinity chromatography to isolate apoC-III from radiolabeled lipoproteins for direct determination of specific radioactivity, we have studied the metabolism of human apoC-III in VLDL and in HDL following the bolus injection of ^{125}I-labeled VLDL. Transfer of apoC-III radioactivity from VLDL to HDL was detected in the plasma sample drawn 5 min after injection of the tracer. However, the specific radioactivity of apoC-III in VLDL was found to be higher than that in HDL, with this difference being maintained throughout the sampling period (48–72 hr). The ratios of the respective specific activities ranged from 1.2 to 1.9 in six subjects studied (two normolipidemics and four hypertriglyceridemics). When ^{125}I-labeled HDL was injected as the tracer, however, the higher apoC-III specific radioactivity was associated with the HDL fraction. This lack of complete equilibration of apoC-III between VLDL and HDL in vivo was further characterized by in vitro studies using either ^{125}I-labeled VLDL or ^{125}I-labeled HDL. All incubations were carried out for 3 hr at 37°C followed by 16 hr at 4°C and the apoC-III specific activity in each lipoprotein fraction was directly determined after immunoaffinity chromatography. In a study of plasma from a mildly hypertriglyceridemic subject in which ^{125}I-labeled VLDL was incubated with unlabeled HDL, apoC-III specific activities in VLDL remained 30% greater than that in HDL. When ^{125}I-labeled HDL (from the same subject) was incubated with unlabeled VLDL of apoC-III, final specific activity in VLDL was less than 10% of that of HDL apoC-III. Differences in specific activities were also demonstrated when radiolabeled purified apoC-III was exchanged onto VLDL prior to its incubation with HDL. A consistent difference in apoC-III specific activities in VLDL and HDL was observed after isolation of the particles either by molecular sieve chromatography or by ultracentrifugation. These studies demonstrated that, while the exchange of apoC-III between VLDL and HDL may be very rapid, this equilibration is not complete. Pools of apoC-III that do not participate in the equilibration process are present in both the VLDL and HDL fractions and could account for 30–60% of the total apoC-III mass in each lipoprotein fraction. — **Bukberg, P. R., N-A. Le, H. N. Ginsberg, J. C. Gibson, A. Rubinstein, and W. V. Brown.** Evidence for non-equilibrating pools of apolipoprotein C-III in plasma lipoproteins. *J. Lipid Res.* 1985. **26:** 1047–1057.

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The C apolipoproteins of human plasma, designated apoC-I, apoC-II, and apoC-III, are small polypeptides of molecular weight 6000–9000. ApoC-III, a single chain polypeptide of 79 amino acids, is found in both nonglycosylated (apoC-III_0) and glycosylated isoforms containing either one (apoC-III_1) or two (apoC-III_2) moles of sialic acid (1, 2). Although the exact metabolic role of apoC-III remains unclear, recent evidence suggests that it may exert a significant effect on the interaction of lipoprotein particles with cell membranes. In particular, studies by Shelburne et al. (3) and Windler, Chao, and Havel (4) have demonstrated pronounced inhibition of the apoE-mediated hepatic clearance of chylomicrons and triglyceride emulsions in the rat following the incubation of these particles with excess apoC-III in vitro. There is also evidence that apoC-III may exert an inhibitory effect on the hydrolytic action of lipoprotein lipase (5) and hepatic triglyceride lipase (6).

As a group, the C apolipoproteins are associated predominantly with particles in the very low density (VLDL, d < 1.006 g/ml) and high density (HDL, d 1.063–1.21 g/ml) lipoprotein classes. Following heparin-induced lipolysis, there is redistribution of apoCs from VLDL to HDL (7). During alimentary lipemia, on the other hand, the C apolipoproteins were shown to be shifted from HDL to newly secreted triglyceride-rich lipoproteins (8). When ^{125}I-labeled VLDL is incubated with plasma in vitro, a rapid redistribution of radioactivity takes place with transfer of radiolabeled apoC primarily to HDL (9). The reverse of this exchange process occurs when radiolabeled HDL is added to plasma (10). Rapid equilibration

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; HDL, high density lipoproteins; LDL, low density lipoproteins; SA, specific activity; IgG, immunoglobulin G; TG, triglyceride; EDTA, ethylene-diamine tetraacetic acid; SDS-PAGE, sodium decyl sulfate polyacrylamide gel electrophoresis.

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of apoC radioactivity has also been demonstrated to occur in vivo following the injection of labeled VLDL or HDL (9, 10). Because the redistribution of apoC radioactivity among lipoprotein particles of the various density classes is nearly instantaneous, it has commonly been assumed that complete equilibration of these apolipoproteins is achieved. No direct support for this assumption, however, has been documented.

Rubenstein and Rubinstein (11) studied phospholipid exchange in vitro using rat plasma and reported the existence of specific and nonspecific processes for phospholipid transfer. Eisenberg and co-workers (12, 13) also investigated the redistribution of various components of rat VLDL during in vitro lipolysis using bovine milk lipoprotein lipase. Similar to the earlier report (11), they also found complexities in the phospholipid exchange process. They noted that the rate of redistribution of ^{32}P-labeled phospholipids was initially slow, but accelerated significantly when more than 40% of the VLDL triglyceride was hydrolyzed (12). A similar nonlinear relationship between the extent of VLDL hydrolysis and the rate of redistribution of ^{125}I-labeled apoC among lipoprotein density fractions was obtained (12). When the dynamics of the exchange of apoC-II and apoC-III$_1$ were examined individually in the same system in an attempt to explain the nonlinear rates of redistribution, no preferential removal of apoC-II or apoC-III$_1$ from lipolyzed VLDL particles could be demonstrated (13). While no explanation was proposed for this observation, it was clear that the rapid redistribution of apoC radioactivity among the lipoprotein particles is not a simple process.

Tracer methods that provide data in terms of total radioactivity only delineate the metabolic fate of the injected material. The ability to obtain experimental data in terms of specific activity, however, provides kinetic information regarding both the disappearance of the injected label as well as the flux of unlabeled material (the tracee). For apoC-III, its specific activity in plasma following the bolus injection of a labeled lipoprotein fraction would depend only on the total apoC-III radioactivity in plasma and the plasma apoC-III pool. Assuming that apoC-III equilibrates completely among all plasma lipoprotein particles, this same value would be obtained for the specific activity of apoC-III associated with any lipoprotein density fraction. Regardless of whether the redistribution of apoC-III among plasma lipoproteins is the result of instantaneous exchange of surface components or hydrolysis of the triglyceride-rich lipoproteins by the lipolytic enzymes or a combination of these two mechanisms, the final specific activity of apoC-III should not be affected.

Recently, using isoelectric focusing to isolate apoC-III from timed plasma samples following the injection of radiolabeled VLDL, Huff et al. (14) observed a 5–10% difference in apoC-III specific activity between VLDL and HDL. The specific activity of apoC-III in this report was based on the relative absorbances of the protein band on scanned electrophoretic gels and measurement of radioactivity from the corresponding gel slices. With the lack of sensitivity of this method, however, they did not regard the differences in apoC-III specific activity between VLDL and HDL as statistically significant and assumed it to be compatible with complete equilibration of apoC-III. We have recently described an alternate method for the direct determination of apoC-III specific activity using immunoaffinity chromatography (15). In our initial turnover studies with radiolabeled VLDL, we found the specific activity of apoC-III in VLDL to be uniformly higher than in HDL, suggesting incomplete in vivo equilibration. This lack of complete equilibration appeared to be more pronounced in normolipidemic individuals than in hypertriglyceridemic subjects. We have since carried out further studies both in vivo and in vitro that provide strong evidence for the presence of non-equilibrating pools of apoC-III in both VLDL and HDL. While the nature of this incomplete equilibration is not clear, its effect upon the in-depth analysis and interpretation of apoC-III kinetic data may be significant and cannot be overlooked.

METHODS

Determination of apoC-III specific radioactivity

The validation of the use of immunoaffinity chromatography in the isolation of apoC-III for specific activity determination has been presented in detail elsewhere (15). In brief, IgG specific for apoC-III was first purified by adsorption of goat antiserum with apoC-III–Sepharose. The purified IgG was then covalently bound to CNBr-activated Sepharose CL-4B (5.5 mg/ml of gel). Nonspecific binding sites were saturated prior to initial use of the gel by incubation with ovalbumin (0.1% solution) followed by extensive washing with 0.2 M glycine (pH 2.5). The binding capacity of the anti-apoC-III–Sepharose was approximately 80–90 μg of apoC-III per ml of gel (15). Contamination of the eluate by other plasma proteins was negligible (15) as demonstrated by a number of methods including SDS-PAGE (for IgG, ovalbumin, and apoA-II), specific radioimmunoassays (for apoB, apoE, and apoA-I) and isoelectric focusing (for apoC-II).

For the isolation of apoC-III from VLDL (20–40% of protein as apoC-III), aliquots containing 250–400 μg of total VLDL protein were delipidated prior to immunoaffinity chromatography. For HDL (with only 3–6% of total protein as apoC-III), larger aliquots containing 1.5–2.0 mg of total protein were required. In addition, the HDL samples were first dialyzed against 1000 volumes of 0.9% NaCl (containing 1 mg/ml EDTA),

and then dried under a stream of nitrogen prior to delipidation. Delipidation of both the VLDL and HDL aliquots was carried out by sequential extraction with acetone and isopropanol, and the protein pellet was resolubilized in borate buffer (0.1 M NaCl, 0.1 M borate, pH 8.0) containing 0.02% Triton X-100. The suspensions were incubated overnight (4°C) to maximize resolubilization and centrifuged at 10,000 rpm for 20 min in microfuge tubes (Microfuge-B, Beckman Instruments, Palo Alto, CA) to remove aggregated material (mostly apoB in VLDL) prior to chromatography over the affinity columns.

The resolubilized apolipoprotein mixtures were applied to individual immunoaffinity columns containing 1 ml of anti-apoC-III–Sepharose (disposable plastic Econocolumns from Bio-Rad, Richmond, CA). Eighteen separate columns were processed at the same time for the 18 timed samples of the turnover studies. Following application of the apolipoprotein solutions to the columns, the columns were washed exhaustively with additional borate buffer to remove nonspecifically bound protein, and the apoC-III was then eluted with 0.2 M glycine, pH 2.5 (elution volume 2.5 ml). For determination of specific radioactivity, measured aliquots of the apoC-III eluate were counted directly in a Packard Autogamma Spectrometer (Packard Instruments, Downers Grove, IL). Protein mass was determined on aliquots of the same eluate by a modification of the Lowry procedure (16, 17) and by radioimmunoassay (15). The columns were then extensively washed using 3 M sodium thiocyanate and reequilibrated in borate buffer before the HDL samples were applied.

The reproducibility of the method has been demonstrated using multiple aliquots of the same radiolabeled lipoproteins at different protein loads. The standard deviation for apoC-III specific activity was less than 6% (15).

In vivo studies

Studies of apoC-III turnover following the injection of ^{125}I-labeled VLDL were carried out in six subjects, two with normal lipoprotein levels and four with Type IV hyperlipoproteinemia. One subject (subject 6) received simultaneous injections of ^{131}I-labeled VLDL and ^{125}I-labeled HDL. Clinical data on the six subjects are given in **Table 1.** Informed consent was obtained from each subject prior to the study.

The turnover studies were carried out in the Mount Sinai Medical Center General Clinical Research Center using a protocol previously described (18). All subjects were stabilized for 4–7 days on a weight-maintaining diet prior to the study (45% carbohydrate, 40% fat, and 15% protein, P/S ratio of 0.4, cholesterol content 150 mg/1000 kcal). Fasting plasma was obtained for the isolation of VLDL (d 1.006 g/ml) and HDL (d 1.063–1.21 g/ml) by ultracentrifugation (19). Both VLDL and HDL were radioiodinated using a modification of the iodine monochloride method (18, 20). All procedures were performed using strict aseptic techniques.

Each subject received a saturated solution of potassium iodide, three drops thrice daily, starting 1 day prior to injection of radiolabeled VLDL and continuing for 3 weeks. A fat-free liquid formula diet was given in equal portions every 3 hr for the duration of the turnover study to prevent possible contamination by chylomicrons (21). Following the injection of 50 μCi of ^{125}I-labeled VLDL (or 50 μCi of ^{131}I-labeled VLDL and 40 μCi of ^{125}I-labeled HDL in subject 6), 17 timed plasma samples (5, 15, 30 min, 1, 1.5, 2, 4, 6, 9, 12, 18, 24, 30, 39, 48, 60, and 72 hr) were obtained. VLDL were isolated from these samples by ultracentrifugation at density 1.006 g/ml, and HDL at density 1.063–1.21 g/ml, using a 40.3 rotor at 39,000 rpm for 20 hr (VLDL) or 48 hr (HDL) at 10°C.

Following the bolus injection of radiolabeled lipoproteins, the specific activity curves for apoC-III VLDL and HDL were biexponential for at least 72 hr. The decay curves were fitted to a sum of two exponentials of the form:

$$A_1 \exp(-b_1 t) + A_2 \exp(-b_2 t).$$

The apparent plasma residence times (15, 22) for apoC-III in each subject were then determined (see Table 2). Estimates of fractional clearance rates for apoC-III in plasma could be obtained as the inverse of the residence time.

In vitro studies

A number of experimental protocols were carried out to characterize the equilibration of apoC-III between VLDL and HDL in vitro. Either radiolabeled whole lipoproteins or radiolabeled apoC-III were used in these studies.

Dynamics of apoC-III in sequential incubation. Plasma was obtained from a patient with Type IV hyperlipoproteinemia (subject 3). Identical aliquots of VLDL and HDL were isolated by ultracentrifugation of 3 ml of plasma in multiple replicates at density 1.006 g/ml and 1.063–1.21 g/ml, respectively, in a 40.3 rotor. Aliquots of

TABLE 1. Clinical characteristics of volunteers

Subject	Age	Plasma Triglyceride	Plasma Cholesterol	HDL Cholesterol
	yr		*mg/dl*	
1	53	49	174	34
2	29	72	128	35
3	57	360	200	25
4	65	466	219	32
5	53	487	231	22
6	65	730	213	17

VLDL and HDL used in the various incubation steps were such that the distribution of apoC-III mass between VLDL and HDL was identical to that found in the original plasma; that is, 493 μg of VLDL apoC-III per ml of plasma versus 88 μg of HDL apoC-III per ml of plasma.

Radiolabeled VLDL. Whole VLDL and HDL were radioiodinated using the iodine monochloride method (9, 20). A tracer amount of autologous ^{125}I-labeled VLDL (less than 10 μg of total protein) was added to individual VLDL samples (1 ml with 493 μg of apoC-III/ml) and incubated at 37°C for 30 min before the addition of the appropriate HDL aliquots (1 ml with 88 μg of apoC-III). The incubation was continued at 37°C for an additional 3 hr and then allowed to proceed at 4°C overnight (16–20 hr). The incubation mixtures were quantitatively transferred to individual tubes for ultracentrifugal fractionation (40.3 rotor). Only a single spin at d 1.020 g/ml was carried out, and the VLDL supernate was recovered quantitatively in 2-ml volumetric flasks. The middle 1 ml was discarded and the 3-ml infranate representing HDL was saved. Aliquots of VLDL supernates and HDL infranates were set aside for apoC-III specific activity determination (incubation A). The ^{125}I-labeled VLDL supernates that were not used for specific activity determination were then combined with fresh unlabeled HDL aliquots (1 ml with 88 μg of apoC-III) for a second incubation (37°C for 3 hr and 4°C overnight) and fractionated as before (incubation B). After this second incubation, aliquots of VLDL supernates and HDL infranates were again set aside for specific activity determination. In the third stage (incubation C), ^{125}I-labeled VLDL fractions that had undergone two successive incubations with fresh HDL were allowed to equilibrate further with additional identical aliquots of fresh unlabeled HDL. In summary, these samples (n = two or three for each set) had been exposed to one, two, or three separate and sequential incubations with identical quantities of unlabeled HDL. The HDL fractions isolated after these incubations with ^{125}I-labeled VLDL were also used to determine apoC-III specific activity.

Radiolabeled HDL. In this experiment, ^{125}I-labeled HDL was added to autologous HDL samples (subject 3) and incubated with unlabeled VLDL samples as just described for the ^{125}I-labeled VLDL and HDL incubations. The quantities of apoC-III in the HDL and VLDL were the same as in the experiment described above, and fresh unlabeled VLDL samples were used at each stage.

In these studies, the concentrations of the two lipoprotein fractions in the incubation mixtures were maintained at levels present in the original plasma. This was designed to prevent the net transfer of apoC-III mass that was noted when an increasing mass of unlabeled lipoproteins was used to deplete radioactivity from the labeled apoC-III pool (data not shown).

ApoC-III labeled by exchange. Available data from our studies indicated that apoC-III specific activity was consistently higher in the lipoprotein first labeled and then added as the source of tracer than in the lipoprotein initially unlabeled but participating in the exchange of labeled apoC-III. This observation suggested that an artifact of the labeling procedure might be involved. It might be postulated that radioiodination could alter certain apolipoprotein molecules causing them to remain more tightly bound to the lipoprotein particles than other molecules, thus resulting in the lack of complete equilibration that was observed.

To test this hypothesis, ^{125}I-labeled HDL isolated from a mildly hypertriglyceridemic donor (TG, 295 mg/dl; HDL cholesterol, 22 mg/dl) was allowed to equilibrate with autologous VLDL at a mass ratio of apoC-III identical to that present in the original plasma. The incubation conditions were identical to those described in the preceding section. The resulting VLDL aliquots, which had acquired apoC-III radioactivity solely by exchange, were then allowed to equilibrate with the appropriate aliquot of unlabeled autologous HDL (again preserving the apoC-III mass ratio present in the original plasma). As in experiments 1a and 1b described above, incubations were carried out in triplicate. Each replicate was handled as a separate sample for the lipoprotein fractionation and the individual lipoprotein fractions thus isolated were delipidated and applied to different affinity columns for apoC-III specific activity determination as separate samples. The replicate apoC-III fractions were each counted for radioactivity and the protein mass from each column eluate was measured in triplicate by the method of Lowry et al. (16) as well as by apoC-III radioimmunoassay (15).

Effect of different methods of lipoprotein isolation. ApoC-III$_2$, prepared by standard chromatographic techniques, was iodinated with ^{125}I using lactoperoxidase (23) and purified as previously described (15). An aliquot of this tracer was added to 10 ml of plasma from a different mildly hypertriglyceridemic subject (TG, 258 mg/dl; HDL cholesterol, 64 mg/dl). The mixture was incubated at 37°C for 3 hr and at 4°C overnight. Lipoprotein fractions were then isolated from aliquots of this mixture by three different methods. *Method A*: Isolation of VLDL (d < 1.006 gm/ml) and HDL (d 1.063–1.21 gm/ml) by sequential ultracentrifugation in a 40.3 rotor (19). *Method B*: Ultracentrifugation in an SW-40 rotor using a single spin discontinuous gradient (24). *Method C*: Gel permeation chromatography of plasma over 4% agarose as previously described (25).

Since only apoC-III was labeled in this experiment, the specific activity could be determined in VLDL and HDL by direct gamma counting of a measured aliquot and determination of apoC-III mass by radioimmunoassay.

RESULTS

In vivo studies

ApoC-III specific activity curves displayed a biphasic decay in all subjects studied. The upper half of **Fig. 1** illustrates typical apoC-III specific radioactivity decay curves for VLDL and HDL obtained following the injection of ^{131}I-labeled VLDL (subject 6). In all studies in which radiolabeled VLDL was the injected tracer, the specific activities of apoC-III in VLDL were always higher than corresponding values for apoC-III in HDL and remained constant throughout the sampling period (**Fig. 2**). The ratios of the specific activities in the two normolipidemic subjects were higher than the values obtained for individuals with hypertriglyceridemia (see **Table 2**). In subject 6 who received both ^{131}I-labeled VLDL and ^{125}I-labeled HDL (see lower half of Fig. 1), the ratios of VLDL apoC-III specific activity to HDL apoC-III specific activity were 1.19 for the VLDL tracer and 0.55 for the HDL tracer (see Table 2).

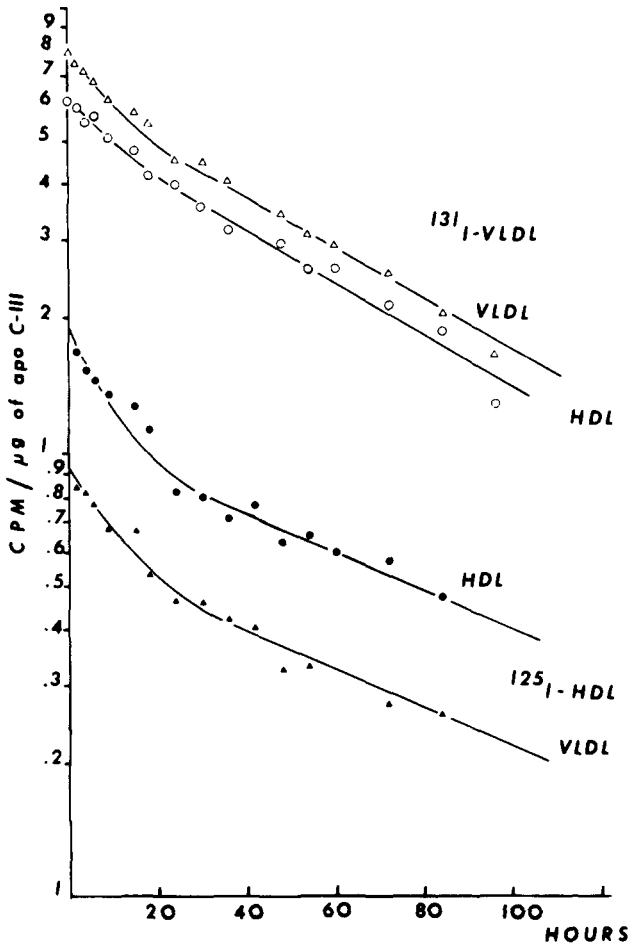

Fig. 1. Apolipoprotein C-III specific activity (SA) decay curves in subject 6 following the injection of ^{131}I-labeled VLDL (open symbols) and ^{125}I-labeled HDL (closed symbols). After injection of the radiolabeled VLDL tracer, the SA curve for VLDL apoC-III (△) was consistently higher than that for HDL apoC-III (○). In contrast, when the tracer was radioiodinated HDL, the HDL apoC-III SA curve (●) was consistently higher than the curve for VLDL apoC-III (▲).

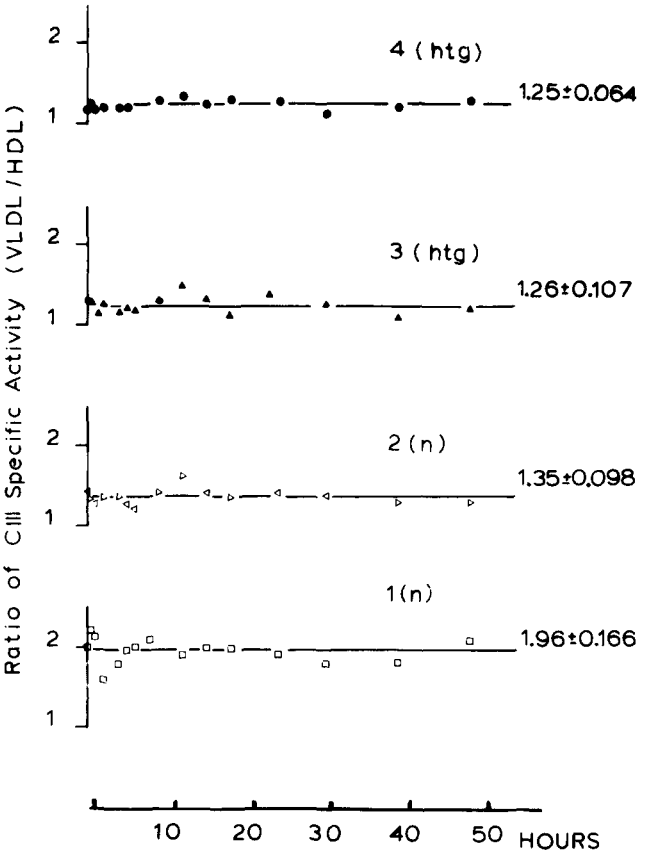

Fig. 2. Ratios of VLDL apoC-III specific activity (SA) to HDL apoC-III SA. At each time point, the ratios of the observed apoC-III SA in VLDL and HDL were determined in four individuals. Subjects 1 and 2 were normolipidemic (n), while subjects 3 and 4 had hypertriglyceridemia (htg). The mean ratio (± SD) of the SA obtained throughout the sampling period (n = 15 samples) was calculated for each subject.

This difference in specific activity for apoC-III between the two lipoprotein fractions was observed in the 5-min sample following the tracer injection of either radiolabeled VLDL or radiolabeled HDL. This finding clearly suggested that while the in vivo redistribution of apoC-III may be essentially instantaneous, this process was not complete, and a true equilibration did not occur.

No significant difference in any of the decay characteristics between VLDL apoC-III and HDL apoC-III specific activity curves was obtained in any subject studied (see Table 2).

In vitro studies

Sequential incubations of whole labeled lipoproteins with unlabeled lipoproteins. Differences in apoC-III specific activity between VLDL and HDL similar to those observed during in vivo studies were also observed in vitro follow-

TABLE 2. Kinetics of apolipoprotein C-III

Subjects			Exponential Components				Residence Time	SA_{VLDL}/SA_{HDL}[a]
			A_1	b_1	A_2	b_2		
			%	hr^{-1}	%	hr^{-1}	hr	
1		V	60.9	0.244	39.1	0.030	15.7	1.35 ± 0.10
		H	46.1	0.258	53.9	0.035	17.0	(n = 15)
2		V	47.1	0.275	52.9	0.025	22.8	1.96 ± 0.17
		H	46.1	0.264	53.9	0.025	23.7	(n = 14)
3		V	21.4	0.210	78.6	0.018	45.9	1.25 ± 0.06
		H	16.0	0.210	84.0	0.018	48.8	(n = 14)
4		V	18.1	0.240	81.9	0.014	61.4	1.16 ± 0.14
		H	19.3	0.261	80.7	0.014	59.2	(n = 13)
5		V	51.5	0.183	48.5	0.011	46.9	1.26 ± 0.11
		H	37.9	0.332	62.1	0.014	44.9	(n = 15)
6[b]	a	V	24.4	0.087	75.6	0.013	61.9	1.19 ± 0.06
		H	12.9	0.091	87.1	0.012	76.5	(n = 13)
6	b	V	24.3	0.093	75.6	0.013	60.7	0.55 ± 0.06
		H	33.3	0.099	66.7	0.010	72.8	(n = 13)

[a]The ratio of the apoC-III specific activity in the two lipoprotein fractions was calculated for each timed sample. The mean values are presented here with the standard deviation and the number of samples used in the computation.

[b]In subject 6, ^{131}I-labeled VLDL was injected simultaneously with ^{125}I-labeled HDL. The data in 6a are derived from the apoC-III specific activity decay curves based on the injection of ^{131}I-labeled VLDL. The data in 6b are based on the injection of ^{125}I-labeled HDL. Only ^{125}I-labeled VLDL was injected in subjects 1–5.

ing the incubation of radiolabeled whole VLDL with unlabeled HDL. The exchange of total radioactivity between VLDL and HDL in vitro (37°C) was complete within the first minute as determined by dextran–magnesium precipitation (data not shown) (18). However, even if the incubation was continued for an additional 3 hr at 37°C followed by 16 hr at 4°C, complete equilibration of the apoC-III radioactivity between VLDL and HDL was not achieved. The specific activity remained higher in VLDL with final values of 205 cpm/μg and 155 cpm/μg for VLDL and HDL apoC-III specific activity, respectively (**Table 3**). When the radiolabeled VLDL isolated from this first incubation was subsequently allowed to equilibrate with fresh unlabeled HDL, the specific activities of apoC-III in the two lipoprotein fractions remained different: 187 cpm/μg for VLDL compared to 110 cpm/μg for HDL. After the third incubation step, the specific activity of apoC-III in VLDL decreased minimally (177 cpm/μg), while the specific activity in HDL was significantly lower (90 cpm/μg) than that in HDL after the second incubation. This corresponded to a transfer of 8.3% of the VLDL apoC-III radioactivity to HDL during the final incubation.

When radiolabeled whole HDL was used as a source of

TABLE 3. Sequential incubations using whole radiolabeled lipoproteins

	^{125}I-Labeled VLDL[a]			^{125}I-Labeled HDL[b]		
	A	B	C	A	B	C
Total radioactivity in apoC-III (cpm)						
VLDL	101,065	91,205	87,261	19,720	4,437	2,465
HDL	13,795	9,680	7,920	43,316	22,000	23,760
% Transferred	12.0	9.6	8.3	31.3	16.8	9.4
Specific activity of apoC-III (cpm/μg)						
Calculated[c]	198	174	164	79	45	45
Observed:						
VLDL	205	187	177	40	9	5
HDL	155	110	90	490	250	270

[a]Incubations with ^{125}I-labeled VLDL containing 493 μg of apoC-III and autologous HDL containing 88 μg of apoC-III. Incubaiton A was the initial incubation. For incubation B, the supernate (^{125}I-labeled VLDL) from incubation A was added to unlabeled HDL containing 88 μg of apoC-III. The ^{125}I-labeled VLDL supernate from incubation B was then added to unlabeled HDL (88 μg of apoC-III) for incubation C.

[b]Incubations with ^{125}I-labeled HDL containing 88 μg of apoC-III with autologous VLDL containing 493 μg of apoC-III. Incubation A was the initial incubation. The HDL fraction from incubation A was added to unlabeled VLDL (493 μg of apoC-III) in incubation B. For incubation C, the HDL fraction from B was used with unlabeled VLDL.

[c]The calculated specific activity was derived from the ratio of the total apoC-III radioactivity added and the total apoC-III mass present in the incubation mixture before separating the lipoprotein fractions.

labeled apoC-III in incubations with unlabeled VLDL, the differences in apoC-III specific activities in the two lipoprotein fractions were even more dramatic with values of 490 and 40 cpm/μg for HDL and VLDL, respectively (see Table 3). With subsequent incubations apoC-III specific activity in HDL seemed to reach a plateau at 250 and 270 cpm/μg while little radioactivity was transferred to VLDL; the values obtained for VLDL apoC-III specific activity were 9 and 5 cpm/μg, respectively. The HDL apoC-III radioactivity transferred to VLDL with each incubation was 31.3, 16.8, and 9.4%, respectively.

If we were to assume that the equilibration of apoC-III between VLDL and HDL was complete, we can determine the expected specific activity in the two fractions by dividing the total apoC-III radioactivity by the total apoC-III mass present in the incubation mixture. From the sequential incubations using radiolabeled VLDL (Table 3), the observed VLDL apoC-III specific activities were somewhat higher than the expected values for each of the three incubations (205 vs 198, 184 vs 174, and 177 vs 164 cpm/μg for incubations A, B, and C, respectively). The corresponding HDL apoC-III specific activities for all three incubation steps on the other hand were significantly lower than the expected values (155 vs 198, 110 versus 174, and 90 versus 164 cpm/μg for incubations A, B, and C, respectively). Together, these results would suggest that the majority of the radiolabeled apoC-III in VLDL participated in the exchange process while a significant portion of the HDL apoC-III mass could not be labeled by exchange. This conclusion was further confirmed in the reverse experiments when radiolabeled HDL from the same subject was used in a series of incubations with unlabeled VLDL (see Table 3). The HDL apoC-III specific activities from these incubations were at least 5-fold greater than the expected values. This would be compatible with the conclusion that only a minor fraction of the HDL apoC-III was exchangeable. Thus, with the majority of the HDL apoC-III radioactivity remaining with the HDL fraction, the resulting specific activities in HDL were significantly higher than those calculated based on the assumption of complete equilibration.

ApoC-III in lipoproteins labeled by exchange. Radioiodinated HDL with an apoC-III specific activity of 69.2 cpm/μg was allowed to equilibrate with an aliquot of autologous VLDL. The individual lipoprotein fractions used in these incubations were isolated from plasma as described in the preceding section, and with each step the respective concentrations of apoC-III from each lipoprotein class were maintained identical to plasma concentrations. Only 1.6% of the total radioactivity initially in HDL was transferred to VLDL with a resulting apoC-III specific activity of 2.2 cpm/μg in VLDL. The final specific activity for apoC-III in HDL was 63.0 cpm/μg. Upon reincubation of this exchange-labeled VLDL sample with an appropriate quantity of fresh unlabeled HDL, 52.3% of the radioactivity was transferred to HDL. The final apoC-III specific activities after this latter incubation were 1.7 cpm/μg in VLDL and 0.9 cpm/μg in HDL.

Effect of the method of lipoprotein isolation. In the final in vitro experiment, we examined the equilibration of purified ^{125}I-labeled apoC-III tracer with the unlabeled apoC-III pool present in whole plasma. The plasma concentrations of apoC-III in the three lipoprotein fractions were 135 μg/ml, 86 μg/ml, and 125 μg/ml for VLDL, IDL + LDL, and HDL, respectively. We utilized three different techniques to isolate the lipoprotein fractions after equilibration of ^{125}I-labeled apoC-III with plasma in order to assess whether the apparent nonequilibration was simply an artifact of the sequential ultracentrifugation procedure utilized in the previous studies. Isolation of VLDL and HDL following the addition of ^{125}I-labeled apoC-III to whole plasma resulted in a consistently higher specific activity for apoC-III in HDL (23.9–24.7 cpm/μg) than in VLDL (14.0–14.8 cpm/μg). More importantly, the specific activity differences were found to be very similar whether the fractions were isolated by sequential ultracentrifugation in a 40.3 rotor, ultracentrifugation in a single-spin discontinuous gradient, or chromatography of plasma over 4% agarose (**Table 4**). ApoC-III specific activities in the IDL and LDL fractions were also similar to each other, but differed from the specific activities in the other two lipoproteins.

DISCUSSION

The observation that radiolabeled C apolipoproteins transfer between VLDL and HDL was first made by Bilheimer, Eisenberg, and Levy (9, 10). The transfer of radioactivity from labeled to unlabeled lipoprotein occurred almost instantaneously and was essentially complete within minutes. At the time there were no practical methods available to these investigators for direct determination of specific activity of the individual C apolipoproteins. This precluded documentation that true and

TABLE 4. Effect of different methods of lipoprotein fractionation

Method	ApoC-III Specific Activity (cpm/μg)		
	VLDL	IDL + LDL	HDL
Sequential ultracentrifugation	14.8	20.8	24.3
Gradient ultracentrifugation	14.0	22.9	24.7
4% Agarose chromatography[a]	14.4	18.6	23.9

[a]The triglyceride peak and the apoA-I peak were used to define the VLDL and HDL ranges, respectively. The IDL + LDL region was defined as the fractions between these two major peaks.

complete equilibration of radiolabeled apoC between VLDL and HDL had been achieved. Rubenstein and Rubinstein (11) were the first to present data suggesting complexities in the process of phospholipid exchange among rat lipoproteins. Eisenberg and co-workers (12, 13) subsequently noted that the rates of redistribution of both phospholipid and apoC radioactivity were functions of the extent of hydrolysis of the VLDL particles by lipoprotein lipase. This redistribution process appeared not to differentiate between apoC-II and apoC-III (13). No data on apoC-III specific activity, however, was available in these studies.

In a recent study, Huff et al. (14) reported kinetic data in man on apoC-III specific activity in VLDL and HDL following the tracer injection of radioiodinated VLDL. The specific activity in this report was determined by isolation of apoC-III by isoelectric focusing, scanning of the gel for estimation of protein mass, and direct counting of the gel slice for radioactivity. In a few of their subjects, 5–10% differences in apoC-III specific activity were obtained between VLDL and HDL. These differences were assumed to be within the experimental error of the method and were not regarded as significant.

Using immunoaffinity chromatography to isolate apoC-III from the individual lipoprotein samples for specific activity determination, we noted that the specific activities for apoC-III in VLDL and HDL at each time point following injection of ^{125}I-labeled VLDL were not identical. In all studies we have carried out to date, the apoC-III specific activity in VLDL was consistently higher than that in HDL at each time point following injection of radiolabeled VLDL. This difference was apparent at the earliest time point, 5 min after tracer injection. The ratio of the corresponding specific activities in VLDL to those in HDL ranged from 1.2 to 1.9 and remained constant throughout the sampling period. This difference in specific activity is consistent with the existence of nonequilibrating pools of apoC-III in either VLDL or HDL or both.

The observed difference in apoC-III specific activities in VLDL and HDL, however, might have been due to technical artifacts. The higher specific activity in VLDL following the injection of ^{125}I-labeled VLDL could involve a systematic contamination of the apoC-III isolated from HDL by an unlabeled apoprotein. We feel that this possibility has been effectively ruled out by assay of isolated apoC-III for contamination by apoproteins B, E, and A-I (by specific radioimmunoassay), apoC-II (by isoelectric focusing), and apoA-II (by polyacrylamide gel electrophoresis). Finally, consistent differences in the apoC-III specific activities in VLDL and HDL were obtained whether the apoC-III mass in the immunoaffinity isolate was determined by the method of Lowry et al. (16) or by direct apoC-III radioimmunoassay (15) (data not shown).

Alternatively, the observed lack of complete equilibration of the apoC-III might have been due to alterations in the affinity of some of these molecules for the native lipoprotein particles induced by the radiolabeling procedure. Such a possibility was suggested by the fact that the labeled lipoprotein consistently had a higher specific activity than the initially unlabeled lipoprotein. However, in vitro studies presented herein using lipoproteins labeled in the apoC-III moiety by exchange (either from whole labeled lipoproteins or from purified apoC-III tracer) did not produce identical apoC-III specific activities in VLDL and HDL, as would be expected if complete equilibration had been achieved. The possibility that the isolation of the various lipoprotein fractions by ultracentrifugation could affect the redistribution of the available apoC-III radioactivity was also ruled out by a study in which lipoprotein fractions were isolated by agarose gel chromatography. These data clearly suggest that nonequilibrating pools of apoC-III are present in both VLDL and HDL.

In order to directly demonstrate that only a portion of apoC-III radioactivity in whole radiolabeled lipoproteins could be depleted by exchange (in the absence of lipolytic activity), we carried out sequential incubation studies with ^{125}I-labeled VLDL and unlabeled HDL as well as with ^{125}I-labeled HDL and unlabeled VLDL. In these experiments, it would be expected that the specific activity of apoC-III associated with the nonequilibrating pool would not be affected while the specific activity of exchangeable apoC-III would be continually reduced by exposure to unlabeled lipoprotein of the other class. The ratio of the specific activity of apoC-III in the radiolabeled lipoprotein fraction to that of the unlabeled fraction should be increasingly higher with each subsequent incubation since less radioactivity would be available for exchange. When ^{125}I-labeled VLDL was combined with unlabeled autologous HDL, 12% of the apoC-III radioactivity initially in VLDL was transferred to HDL, resulting in a ratio of apoC-III specific activities of 1.32 with the higher specific activity in VLDL. With each sequential incubation of the same ^{125}I-labeled VLDL with fresh unlabeled HDL, the percent of the apoC-III radioactivity which was transferred to HDL decreased, and the ratio of specific activities (VLDL/HDL) increased to a final value of 1.97 by the third incubation.

In incubation studies using ^{125}I-labeled HDL, 31% of the apoC-II radioactivity was transferred in the first incubation, resulting in a ratio of apoC-III specific activity of 12.25. By the third incubation, only 10% of the remaining apoC-III radioactivity was transferred and the difference in specific activity was even more dramatic with a ratio of 54.0. These studies clearly demonstrated the presence of a nonexchangeable pool of apoC-III in both VLDL and HDL and further suggested that, at least in hypertriglyceridemic subjects, this pool may represent the

major portion of HDL apoC-III.

Hypothetical kinetic scheme

While additional kinetic studies will be required to allow the development of a quantitative multicompartmental model for apoC-III metabolism in man, a number of basic features of such a model for apoC-III kinetics in plasma following the injection of radioiodinated VLDL can be delineated (**Fig. 3**). The notations 'E' and 'NE' are used here to denote the equilibrating and nonequilibrating pools of apoC-III, respectively, within each lipoprotein density fraction. These pools may represent different regions on the same lipoprotein particles and/or different subpopulations of particles within the density class. Upon the injection of radiolabeled VLDL, radioactivity associated with the exchangeable pool of VLDL is instantaneously distributed among other exchangeable apoC-III pools in IDL, LDL, and HDL (process 1). Hydrolysis of VLDL triglycerides by plasma lipases may shift the nonexchangeable VLDL apoC-III pool into the (IDL + LDL) density range either as nonexchangeable (process 2) or as exchangeable apoC-III (process 3). During the final stage of VLDL hydrolysis, most of the nonexchangeable apoC-III in the (IDL + LDL) fraction may become exchangeable (process 4). In both instances, (processes 3 and 4), the exchangeable apoC-III in IDL and LDL would then immediately equilibrate with exchangeable apoC-III in both HDL and nascent VLDL. In this schema, process 1 would correspond to the process of instantaneous equilibration while processes 2 through 4 would reflect various stages of lipolysis in vivo.

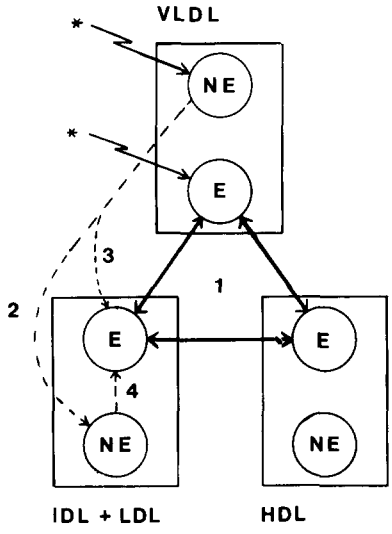

Fig. 3. Schematic illustrating the basic features of apoC-III metabolism in man. Three lipoprotein classes are considered: VLDL (d 1.006 g/ml), IDL + LDL (d 1.006–1.063 g/ml) and HDL (d 1.063–1.210 g/ml). ApoC-III within each density fraction is distributed between an exchangeable pool (E) and a nonexchangeable pool (NE). After the injection of radiolabeled VLDL, both the E and NE pools of VLDL will have initial radioactivity. Radioactivity in the exchangeable VLDL pool is instantaneously distributed among other exchangeable pools (process 1). All exchangeable apoC-III will, therefore, have the same specific activity by the time the first blood sample is obtained (5 min). As a result of lipolysis, VLDL are converted to IDL + LDL. The nonexchangeable apoC-III initially in VLDL may remain nonexchangeable (process 2) or become exchangeable (process 3). Process 4 implies that ultimately all of the apoC-III in apoB-containing lipoproteins will become exchangeable. The apoC-III specific activity obtained for each density class will then depend on the fraction of the plasma exchangeable apoC-III pool that is associated with that class and the size of the nonexchangeable pool of apoC-III in that lipoprotein class. The nonexchangeable apoC-III pool in HDL does not become exchangeable, but does appear to remain associated with the HDL particles as they are irreversibly removed from the circulation.

It should be emphasized that Fig. 3 only attempts to summarize the characteristics of one system that would satisfy our observations from both in vivo and in vitro studies. However, independent of the mechanisms underlying these processes, the lack of complete equilibration of apoC-III between VLDL and HDL is clearly demonstrated. Whether the exchange process is instantaneous, a result of lipolytic actions, or a function of phospholipid transfer protein (26), or any combination of these processes, only one single specific activity for apoC-III should be obtained in each and all of the lipoprotein fractions, if complete equilibration occurs.

Based on this "conceptual model," a number of metabolic pathways can be proposed for apoC-III in man. If we assume that nonequilibrating apoC-III in VLDL will ultimately become part of the plasma pool of exchangeable apoC-III as a result of lipolysis, all of the injected apoC-III radioactivity will be available for participation in the redistribution after hydrolysis. This transition could account for the change in the rate of redistribution of apoC radioactivity reported by Eisenberg et al. (13) with hydrolysis of VLDL by lipoprotein lipase in vitro. The terminal decay component of the specific activity curve would thus reflect the metabolism of plasma exchangeable apoC-III. During this phase, the apoC-III specific activity in each lipoprotein fraction will depend on the specific activity of the plasma pool of exchangeable apoC-III and the relative mass contribution of equilibrating and nonequilibrating apoC-III in that fraction. If we postulate that all of the apoC-III in HDL could equally participate in the redistribution process, we would expect to obtain a higher specific activity for apoC-III in HDL during this phase since the VLDL specific activity would be diluted by continued secretion of unlabeled apoC-III into the nonequilibrating pool. In other words, the kinetic decay curves of apoC-III specific activity would be expected to cross one another during this phase if there is only one pool of apoC-III within HDL. The finding that the two specific activity curves remained parallel in all subjects studied therefore suggests that only a portion of the apoC-III pool in plasma HDL can participate in the redistribution.

Furthermore, our data also imply that the nonequilibrating apoC-III pool in HDL never participates in the

equilibration process. If the radioactivity injected as nonequilibrating HDL apoC-III (for example, from the ^{125}I-labeled HDL injection in subject 6) were to participate in the exchange process after some time in the circulation, we would have expected the HDL and VLDL apoC-III specific activity curves to cross one another, resulting in the same final ratio of specific activity as observed when the injected tracer in that study was whole VLDL. We did not observe such a crossover in our study. As shown in Fig. 1, injection of radioiodinated HDL resulted in a higher HDL apoC-III specific activity throughout the sampling period, with the ratio of the specific activity between HDL and VLDL being different from the ratio obtained when ^{131}I-labeled VLDL was injected in the same individual. In other words, our data would suggest that, unlike in VLDL, apoC-III associated with the nonequilibrating pool of HDL should never become exchangeable. If the assumptions of this scheme are correct, we would also have to postulate that the major pathway for irreversible loss of plasma apoC-III is via HDL.

The concept of nonequilibrating apoC-III pools in different lipoprotein fractions, as well as the necessity to estimate the relative sizes of these pools, is of great significance in any attempt to derive a satisfactory model for apoC-III metabolism. Thus, based on the present data, studies carried out in which radiolabeled pure apoC-III had been allowed to equilibrate onto lipoproteins would reflect only the fractional clearance rate of the common plasma pool of exchangeable apoC-III. This method will completely ignore any flux of apoC-III through the nonexchangeable pools as well as any difference in the distribution of exchangeable and nonexchangeable apoC-III within each density class. While the use of radiolabeled whole lipoproteins would allow the labeling of both equilibrating and nonequilibrating pools, estimates of the kinetic parameters (clearance and production rates) would have to take into account the potential difference in metabolic fates of the two pools. Our finding of a significant pool of apoC-III in the IDL + LDL fraction of some subjects (in particular, those with hypertriglyceridemia), mandates that the specific activity decay curve for apoC-III in this fraction be analyzed also. Preliminary data on the specific activity of apoC-III in the density fraction 1.006–1.063 g/ml, which includes IDL and LDL in two hypertriglyceridemic subjects, indicates a precursor–product relationship for apoC-III between VLDL and (IDL + LDL). The peak apoC-III specific activity was achieved at approximately 4 and 5 hr in these two studies (data not shown). The decaying phase of these apoC-III specific activity curves was intermediate between that of VLDL and HDL apoC-III specific activity. Thus, any attempt to derive a detailed kinetic model for apoC-III metabolism would require specific activity decay curves for VLDL, IDL + LDL, and HDL following injection of both ^{131}I-labeled VLDL and ^{125}I-labeled HDL as well as estimates of the relative distribution of exchangeable and nonexchangeable apoC-III within each lipoprotein density class.

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