Storage of human plasma samples leads to alterations in the lipoprotein distribution of apoC-III and apoE

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Abstract   The effect of frozen storage on lipoprotein distribution of apolipoprotein C-III (apoC-III) and apoE was investigated by measuring apoC-III and apoE by ELISA in HDL and apoB-containing lipoproteins of human plasma samples (n = 16) before and after 2 weeks of frozen storage ($-20°C$). HDLs were separated by heparin-manganese precipitation (HMP) or by fast-protein liquid chromatography (FPLC). Total plasma apoC-III and apoE levels were not affected by frozen storage. HDL-HMP apoC-III and apoE levels were significantly higher in frozen versus fresh samples: 7.7 ± 0.7 versus 6.7 ± 0.7 mg/dl ($P < 0.05$) and 2.0 ± 0.1 versus 1.2 ± 0.1 mg/dl ($P < 0.001$), respectively. HDL-FPLC apoC-III and apoE, but not triglyceride (TG) or cholesterol, levels were also higher in frozen samples: 12.0 ± 1.2 versus 7.5 ± 0.6 mg/dl ($P < 0.001$) and 2.7 ± 0.2 versus 1.6 ± 0.2 mg/dl ($P < 0.001$), respectively. Frozen storage led to a decrease in apoC-III ($-17 \pm 9\%$) and apoE ($-19 \pm 9\%$) in triglyceride-rich lipoprotein. Redistribution of apoC-III and apoE was most evident in samples with high TG levels. HDL apoC-III and apoE levels were also significantly higher when measured in plasma stored at $-80°C$.   Our results demonstrate that lipoprotein distribution of apoC-III and apoE is affected by storage of human plasma, suggesting that analysis of frozen plasma should be avoided in studies relating lipoprotein levels of apoC-III and/or apoE to the incidence of coronary artery disease.—Cohn, J. S., C. Rodriguez, H. Jacques, M. Tremblay, and J. Davignon. **Storage of human plasma samples leads to alterations in the lipoprotein distribution of apoC-III and apoE.** *J. Lipid Res.* 45: **1572–1579.**

Patients with coronary artery disease (CAD) tend to have increased levels of plasma triglyceride (TG) (1–3). However, multivariate analyses of prospective data have often failed to identify plasma TG as an independent risk factor for CAD (4, 5). This is because plasma TG levels are closely related to other lipid (e.g., HDL cholesterol) as well as nonlipid risk factors (e.g., obesity, diabetes, and cigarette smoking) (6). In addition, not all triglyceride-rich lipoproteins (TRLs) are equally atherogenic, and partially catabolized TRLs (i.e., remnant lipoproteins) are believed to promote the onset and development of atherosclerosis to a greater extent than do large, lipid-rich TRLs (7). Studies have therefore been conducted to identify components of large and small TRLs that might be better able to predict risk of CAD.

Apolipoprotein C-III (apoC-III) and apoE are two candidate CAD risk factors that are known to play a key role in regulating plasma TRL metabolism. ApoC-III inhibits TRL catabolism by reducing the lipolysis and uptake of TRL (8, 9). ApoE, on the other hand, enhances TRL catabolism by: *a*) promoting conversion of VLDL to IDL and LDL, and *b*) acting as a ligand for receptor-mediated uptake of TRL remnants (10). Overexpression of human apoC-III in mice thus leads to severe hypertriglyceridemia (11) and increased development of atherosclerosis (12), whereas apoE protects mice from hyperlipidemia and atherosclerotic lesion formation (13). Interestingly, patients with CAD have increased levels of both apoC-III and apoE in their TRL (14), consistent with a direct inhibitory effect of apoC-III on TRL catabolism and an intolerance or insensitivity to the lipid-clearing effects of apoE.

Both angiographic and prospective coronary event studies have demonstrated that increased levels of apoC-III or apoE in TRL (or reduced levels of apoC-III in HDL) are independently associated with the progression or severity of CAD (15–19). In the Cholesterol-Lowering Atherosclerosis Study, the predominant factor predicting the probability of global coronary progression in subjects treated with colestipol plus niacin was a decrease in the concentration of HDL apoC-III (15). In the Monitored Atherosclerosis Regression Study, predominant risk factors for severe lesions in lovastatin-treated patients were LDL cholesterol, the LDL-C to HDL-C ratio, and apoB, while pre-

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Abbreviations: apo, apolipoprotein; CAD, coronary artery disease; FPLC, fast-protein liquid chromatography; HC, hypercholesterolemic; HMP, heparin-manganese precipitation; HTG, hypertriglyceridemic; NL, normolipidemic; TG, triglyceride; TRL, triglyceride-rich lipoprotein.

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dominant risk factors for mild/moderate lesions were TG levels and the concentration of apoC-III in apoB-containing lipoproteins (16). A nested case control analysis of samples from the Cholesterol and Recurrent Events trial found that VLDL-apoB, apoC-III in VLDL + LDL, and apoE in HDL were significant independent predictors of coronary events (18).

The long time course and large number of patients in multicentre studies often require that plasma samples be stored at −20°C or −80°C for later analysis. Therefore, in most of the aforementioned studies (15, 16, 18, 19), lipoprotein distribution of apoC-III and/or apoE was determined in plasma that had been previously frozen and thawed. In contrast, metabolic studies in our laboratory have always involved the measurement of apoC-III and/or apoE in lipoproteins from freshly isolated plasma (20–23). Our experience is that frozen storage does not affect the total plasma concentration of apoC-III and/or apoE, but that it can affect the relative amount of these apolipoproteins in different lipoprotein fractions. To document this effect, we carried out the present study, in which human plasma samples were stored under various conditions and lipoprotein distribution of apoC-III and apoE was determined before and after storage.

METHODS

Samples and storage

Blood samples were obtained after an overnight fast from patients being treated at the lipid clinic of the Clinical Research Institute of Montreal. Patients were selected on the basis of their fasting TG concentration at a previous visit—from low to moderately elevated (<8 mmol/l). They agreed to have their blood used for scientific purposes through signed consent. Blood was drawn from an arm vein into Vacutainer tubes containing EDTA (final concentration, 1.5 mg/ml). Samples were centrifuged immediately (15 min, 3,000 rpm, 4°C). Plasma was collected by aspiration, and samples were immediately immersed in crushed ice. Plasma samples (1.5 ml aliquots) were then stored at 4°C in a Uni-Therm refrigerator (Grand Haven, MI) until analysis (within 4 h) or were frozen by being placed in a Revco −20°C (Asheville, NC) or Baxter −80°C (Asheville, NC) laboratory freezer. In some experiments, plasma samples were rapidly frozen by immersion in liquid nitrogen. After 2 weeks, frozen samples were thawed for analysis by being left at room temperature (1 h, 24°C). In some experiments, samples were thawed by being incubated for 30 min at 37°C.

Separation of lipoproteins

HDLs were separated from apoB-containing lipoproteins by heparin-manganese precipitation (HMP) according to the Lipid Research Clinics procedure (24), as modified by Warnick and Albers (25). Heparin-MnCl$_2$ solution was added to plasma at a ratio of 0.1:1.0 (v/v). After 30 min at room temperature, samples were centrifuged (60 min, 3,000 rpm, 4°C). Supernates were aspirated and stored at 4°C until analysis (<24 h). Lipoproteins were also separated from plasma samples by automated gel filtration chromatography on a Pharmacia (Pharmacia LKB Biotechnology, Uppsala, Sweden) fast-protein liquid chromatography (FPLC) system, as described previously (20, 21). Plasma samples (1 ml) were manually transferred to a 2 ml sample loop with two washes

of 0.5 ml saline solution. They were programmed (Liquid Chromatography Controller LCC-500 Plus) to be loaded and separated on a 50 cm column (16 mm internal diameter) packed with cross-linked agarose gel (Superose 6 prep grade, Pharmacia). The column was eluted with 0.15 mol/l NaCl (0.01% EDTA, 0.02% sodium azide, pH 7.2) at a rate of 1.0 ml/min, and 25 min after addition of sample, 80 × 1 ml fractions were collected sequentially. Total run time for each sample, including pre- and postwashes was 150 min. Sample elution was monitored spectrophotometrically at an optical density of 280 nm. Three peaks of TG or cholesterol were identifiable for each plasma sample, corresponding to TRL, IDL + LDL, and HDL lipoprotein fractions. FPLC profiles were analyzed by measuring analytes in every second tube (from fractions 4–56), or pooled fractions were assayed: TRL (5–14), IDL + LDL (15–35), and HDL (36–56). Recovery of lipoproteins was 80–90%, and all concentrations were corrected to give 100% recovery.

Lipid and apolipoprotein analyses

Plasma and lipoprotein TG and cholesterol concentrations were determined enzymatically on an autoanalyzer (Cobas Mira, Roche). ApoC-III and apoE were measured by ELISA using immunopurified polyclonal antibodies (20, 21).

Statistics

Mean data were compared before and after storage by Student's paired t-test. Means that were different with a probability of less than 5% that this was by chance ($P < 0.05$) were taken to be significantly different.

RESULTS

Plasma samples varied in TG concentration from 0.68 to 7.63 mmol/l and in total cholesterol concentration from 3.82 mmol/l to 8.14 mmol/l. Mean plasma TG concentration of samples (n = 16) before storage was 2.29 ± 0.45 mmol/l, and mean plasma cholesterol concentration was 5.59 ± 0.31 mmol/l. As shown in **Table 1**, frozen storage led to a small (3.9%) but consistent ($P < 0.01$) increase in the measured concentration of plasma TG, as described previously (26). Total plasma cholesterol, apoC-III, and apoE levels were not significantly affected by storage. HDLs were isolated by HMP before and after sample

TABLE 1. Total plasma and HDL lipid and apolipoprotein concentrations in samples before and after frozen storage

	Before Storage	After Storage	Percentage Difference	P^a
Total plasma				
TG	2.29 ± 0.45	2.36 ± 0.46	3.9 ± 1.0	<0.01
Cholesterol	5.59 ± 0.31	5.72 ± 0.31	2.4 ± 1.5	—
apoC-III	22.9 ± 2.7	24.8 ± 3.2	7.9 ± 3.3	—
apoE	5.4 ± 0.4	5.5 ± 0.5	0.1 ± 3.2	—
HDL				
TG	0.26 ± 0.03	0.22 ± 0.02	−9.9 ± 2.8	<0.05
Cholesterol	1.19 ± 0.08	1.18 ± 0.08	−0.5 ± 1.4	—
apoC-III	6.7 ± 0.7	7.7 ± 0.7	25.4 ± 12.2	<0.05
apoE	1.2 ± 0.1	2.0 ± 0.1	99.0 ± 31.1	<0.001

ApoC-III, apolipoprotein C-III; TG, triglyceride. Results represent means ± SE for 16 samples. Lipid levels are expressed in units of mmol/l and apolipoproteins in units of mg/dl. HDLs were separated by heparin-manganese precipitation (HMP).

[a] Statistical significance of difference due to storage.

storage. HDL-HMP cholesterol levels were not affected by storage, but HDL-HMP TG levels were consistently lower (−9.9%) in plasma samples that had been frozen. As predicted, HDL-HMP apoC-III concentrations were significantly higher (25%, $P < 0.05$) in samples after storage. HDL apoE levels were also higher, and this increase (99%) was greater than that for apoC-III. To verify the aforementioned results, lipoproteins were separated by FPLC from the same plasma samples (n = 16) before and after storage. FPLC profiles for two samples are shown in **Figs. 1**, **2**. The plasma in Fig. 1 had TG, cholesterol, apoC-III, and apoE concentrations of 2.30 mmol/l, 4.56 mmol/l, 18.5 mg/dl, and 5.1 mg/dl, respectively. The sample in Fig. 2 had TG, cholesterol, apoC-III, and apoE concentrations of 4.84 mmol/l, 5.56 mmol/l, 47.9 mg/dl, and 6.7 mg/dl, respectively. The sample shown in Fig. 2, therefore, had 2-fold higher TG and apoC-III levels than did that shown in Fig. 1. Frozen storage of these samples had qualitatively similar effects on the lipoprotein distribution of plasma lipids and apolipoproteins. In both cases, there were small increases in TRL TG and TRL cholesterol after storage at the expense of TG and cholesterol in large LDL or remnant particles. No change was observed in the size or position of HDL TG and HDL cholesterol peaks in samples after storage. In contrast, frozen storage had a marked effect on lipoprotein distribution of apoC-III and apoE, with considerably more apoC-III and apoE being detected in HDL and considerably less in TRL and/or IDL/LDL. The magnitude of these changes is depicted by mean data for all samples (n = 16) presented in **Table 2**. Total plasma apoC-III and apoE measurements were not significantly affected by storage. HDL-FPLC apoC-III levels were higher by 65% on average ($P < 0.001$), and HDL-FPLC apoE levels were higher by 104% ($P < 0.001$). At the same time, TRL apoC-III levels decreased by 17% on average ($P < 0.001$), and TRL apoE levels decreased by 19% ($P < 0.05$). IDL/LDL apoC-III and apoE levels also decreased, although only the change in IDL/LDL apoE was statistically significant ($−25\%$, $P < 0.001$). Mean TRL TG concentrations before and after storage were 1.19 ± 0.36 and 1.32 ± 0.40 mmol/l ($P < 0.01$). Mean TRL cholesterol concentrations before and after storage were 0.37 ± 0.12 and 0.50 ± 0.16 mmol/l ($P < 0.01$). Frozen storage therefore caused significant increases in TRL lipids but significant decreases in TRL apoC-III and apoE. Mean HDL-FPLC cholesterol concentrations before and after storage were 1.13 ± 0.08 and 1.13 ± 0.16 mmol/l (not significant, $P = 0.94$). Mean HDL-FPLC TG concentrations before and after storage were 0.19 ± 0.02 and 0.19 ± 0.02 mmol/l (not significant, $P = 0.67$). HDL-FPLC lipid levels were therefore unaffected by storage despite marked increases in HDL-FPLC apoC-III and apoE.
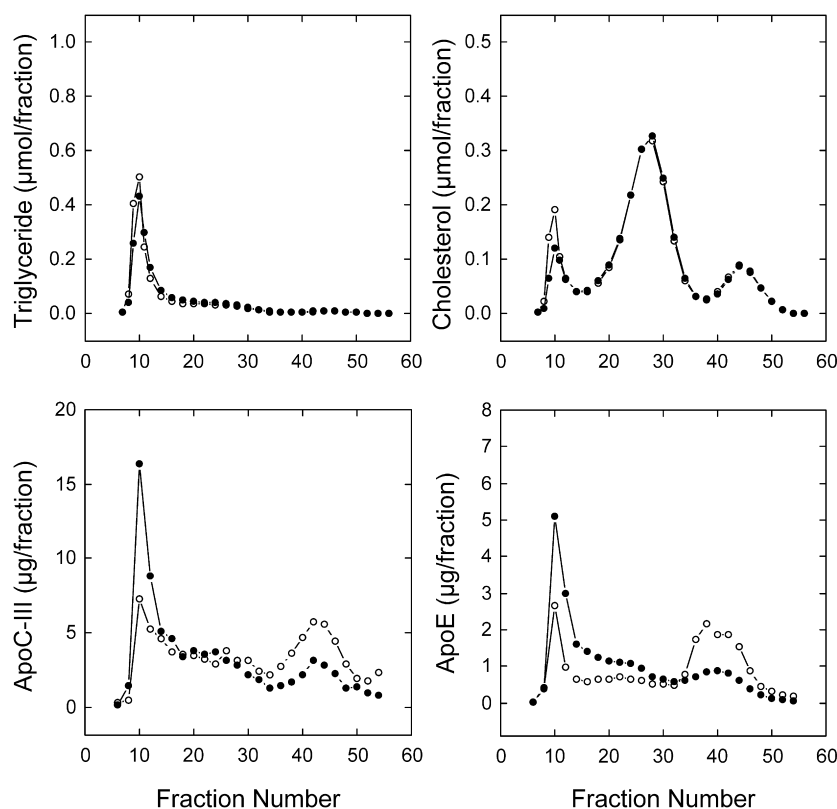
Fig. 1. Separation by fast-protein liquid chromatography (FPLC) of lipoproteins from fresh and frozen plasma of a male subject with a plasma triglyceride (TG) concentration of 2.3 mmol/l and a plasma cholesterol concentration of 4.6 mmol/l. Results for fresh plasma are shown with filled circles, and those for frozen plasma are shown with open circles. Triglyceride and cholesterol data are shown in the upper panels and apolipoprotein data are shown in the lower panels.
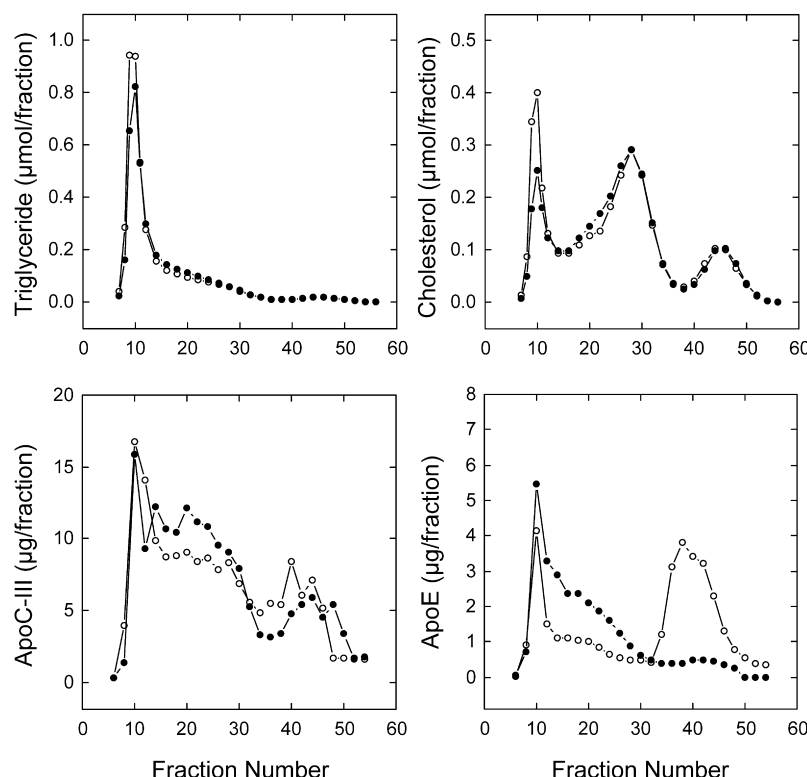
Fig. 2. Separation by FPLC of lipoproteins from fresh and frozen plasma of a male subject with a plasma TG concentration of 4.8 mmol/l and a plasma cholesterol concentration of 5.6 mmol/l. Results for fresh plasma are shown with filled circles and those for frozen plasma are shown with open circles. Triglyceride and cholesterol data are shown in the upper panels and apolipoprotein data are shown in the lower panels.

From the raw data, it was evident that samples with higher TG levels tended to have greater changes in lipoprotein apoC-III and apoE. To show this effect, samples were divided into quartiles on the basis of their TG concentration. Mean TG levels for the quartiles are shown in **Table 3**. Mean plasma apoC-III levels for the four quartiles were 13.9 ± 1.8, 16.3 ± 2.9, 24.2 ± 2.0, and 37.3 ± 4.6 mg/dl, respectively. Mean plasma apoE levels for the four quartiles were 4.4 ± 0.4, 4.2 ± 0.6, 5.4 ± 0.2, and 7.7 ± 0.4 mg/dl, respectively. Apolipoprotein levels determined in frozen samples were then expressed as a percentage of levels found in fresh samples, and mean data for the different quartiles were determined (Table 3). Independent of whether HDLs were separated by HMP or FPLC, samples with higher TG levels had greater increases in HDL apoC-III and apoE. These samples also had greater decreases in TRL apoC-III and apoE. Mean absolute increases in TRL TG for the four quartiles (Q1 to Q4) were 0.01 ± 0.01, 0.06 ± 0.01, 0.10 ± 0.01, and 0.35 ± 0.12 mmol/l, respectively, and mean absolute increases in TRL cholesterol for the four quartiles (Q1 to Q4) were 0.02 ± 0.01, 0.08 ± 0.01, 0.13 ± 0.01, and 0.32 ± 0.08 mmol/l, respectively. Thus, decreases in TRL apolipoproteins were reciprocally related to increases in TRL lipids.

To determine the effect of storage temperature on measured levels of HDL apoC-III and apoE, plasma samples (n = 5) were stored for 2 or 6 days at 4°C, or for 2 weeks at either −20°C or −80°C. Samples were thawed in the usual

fashion at room temperature (24°) for 1 h. Results are shown in **Fig. 3**. As found previously (Table 1), HDL apoC-III and apoE levels were higher in plasma samples after storage at −20°C, and HDL apoE levels tended to increase to a greater extent than did HDL apoC-III levels. Storage at −80°C also resulted in an increase in HDL apoC-III and apoE concentrations. HDL apoC-III levels before and after storage at −80°C were 4.1 ± 0.6 versus 5.5 ± 0.8 mg/dl ($P < 0.05$), and HDL apoE concentrations before and af-

TABLE 2. Total plasma and lipoprotein apoC-III and apoE concentrations in samples before and after frozen storage

	Before Storage	After Storage	Percentage Difference	P^a
apoC-III				
Total	22.9 ± 2.7	24.8 ± 3.2	7.9 ± 3.3%	—
TRL	7.1 ± 1.8	5.2 ± 1.3	−17.1 ± 8.5%	<0.001
IDL/LDL	8.3 ± 1.1	7.6 ± 1.1	−8.0 + 7.1%	—
HDL	7.5 ± 0.6	12.0 ± 1.2	64.6 ± 14.9%	<0.001
apoE				
Total	5.4 ± 0.4	5.5 ± 0.5	0.1 ± 3.2%	—
TRL	1.3 ± 0.3	0.8 ± 0.2	−19.0 ± 9.4%	<0.05
IDL/LDL	2.6 ± 0.3	1.9 ± 0.2	−25.2 ± 4.8%	<0.001
HDL	1.6 ± 0.2	2.7 ± 0.2	103.6 ± 30.4%	<0.001

IDL, intermediate density lipoprotein; TRL, triglyceride-rich lipoprotein. Results represent means ± SE for 16 samples. Apolipoprotein levels are expressed in units of mg/dl. Lipoprotein fractions were separated by fast-protein liquid chromatography (FPLC).

[a] Statistical significance of difference due to storage.

TABLE 3. Effect of frozen storage on apoC-III and apoE levels in samples grouped according to their plasma TG concentration

	Q1	Q2	Q3	Q4	P^a
Plasma TG (mmol/l)	0.76 ± 0.03	1.48 ± 0.16	2.24 ± 0.04	4.68 ± 1.07	<0.001
TRL apoC-III	106 ± 15	88 ± 7	70 ± 2	68 ± 4	—
IDL/LDL apoC-III	77 ± 6	83 ± 8	98 ± 5	110 ± 9	—
HDL apoC-III	123 ± 4	161 ± 11	151 ± 7	224 ± 22	<0.01
HDL-HMP apoC-III	98 ± 4	100 ± 6	137 ± 13	166 ± 16	<0.01
TRL apoE	120 ± 11	77 ± 2	78 ± 8	48 ± 6	<0.01
IDL/LDL apoE	82 ± 2	77 ± 3	83 ± 6	57 ± 6	—
HDL apoE	122 ± 3	144 ± 11	221 ± 13	328 ± 46	<0.01
HDL-HMP apoE	109 ± 5	128 ± 3	242 ± 28	317 ± 41	<0.01

Samples were grouped according to plasma TG concentration and then divided into quartiles (Q). Values represent means ± SE for samples (n = 4) in each quartile. Apolipoprotein levels in frozen samples were expressed as a percentage of levels in fresh samples. Results are shown for HDL separated by FPLC and for HDL separated by precipitation (HMP).

[a] Statistical significance of difference between groups (by ANOVA).

ter storage were 0.6 ± 0.2 versus 1.2 ± 0.2 mg/dl ($P <$ 0.05). Addition of sucrose as a stabilizing agent to plasma samples before freezing [50% solution, 1:20 (v/v)] did not prevent storage-induced increases in HDL apolipotein levels (data not shown). Rapid freezing of plasma samples by immersion in liquid nitrogen tended to reduce storage-induced increases in HDL apolipoprotein levels, but it did not provide complete protection against this effect. HDL apoC-III levels in plasma stored for 2 weeks at −20°C with and without rapid freezing were 5.4 ± 0.6 versus 5.0 ± 0.5 mg/dl ($P = 0.054$); HDL apoC-III levels in freshly isolated samples were 4.1 ± 0.6 mg/dl. HDL apoE levels in plasma stored for 2 weeks at −20°C with and without rapid freezing were 1.5 ± 0.2 versus 1.4 ± 0.2 mg/dl ($P = 0.32$); HDL apoE levels in freshly isolated samples were 0.6 ± 0.2 mg/dl. Thawing samples (n = 5) for 30 min at 37°C, rather than thawing them for 1 h at room temperature (24°C), had no significant effect on HDL apolipoprotein levels, i.e., HDL apoC-III levels after 2 week storage at −80°C thawed for 1 h at 24°C versus 30 min at 37°C were 6.6 versus 6.4 mg/dl ($P = 0.94$, not significant), and HDL apoE levels were 1.3 versus 1.2 mg/dl ($P = 0.47$, not significant). Even at 4°C, less-pronounced yet statistically significant increases were observed in HDL apoC-III and apoE levels after 2 or 6 days of storage, i.e., HDL apoC-III levels at 0, 2, and 6 days were 4.1 ± 0.6, 5.2 ± 0.7, and 5.5 ± 1.1 mg/dl, respectively, $P < 0.05$; HDL apoE levels at 0, 2, and 6 days were 0.6 ± 0.2, 0.9 ± 0.2, and 0.9 ± 0.2 mg/dl, respectively, $P < 0.05$. These changes in HDL apolipoprotein levels occurred independently of any change in levels of total plasma apoC-III, plasma apoE, or HDL cholesterol (HDL cholesterol levels at 0 and 6 days were 0.97 ± 0.05 versus 0.95 ± 0.05 mmol/l, $P = 0.84$, not significant).

DISCUSSION

The present results demonstrate that storage of human plasma samples leads to a significant change in the lipoprotein distribution of apoC-III and apoE. Irrespective of
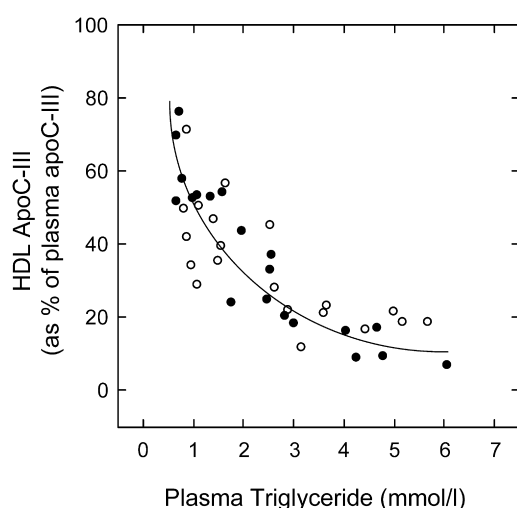
whether HDLs were separated by precipitation (HMP) or size exclusion chromatography, significantly higher levels of apoC-III and apoE were found in HDLs separated from samples stored for 2 weeks at −20°C. Less apoC-III and apoE were recovered in apoB-containing lipoproteins, particularly TRLs. Changes in TRL and HDL apolipoprotein levels were not associated with similar changes in TRL and HDL TG or cholesterol and they were most evident in samples with high TG levels. HDL apoC-III and apoE levels were also significantly higher in plasma samples frozen at −80°C. Even in unfrozen samples (stored at 4°C), less-pronounced yet statistically significant increases were ob-
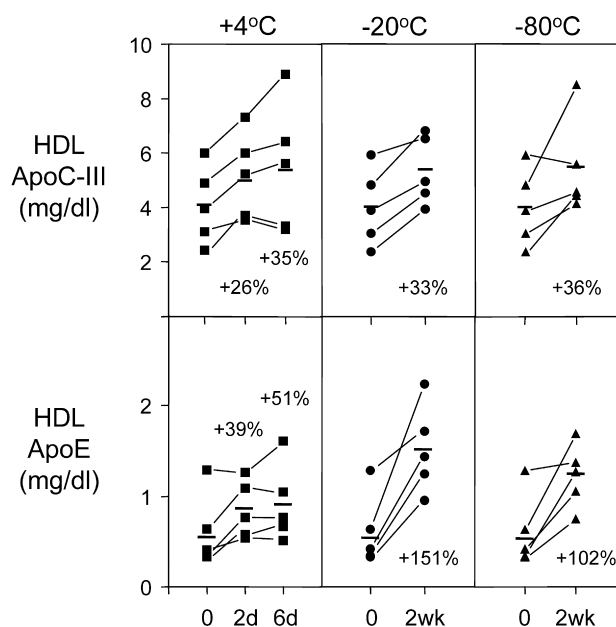
Fig. 3. Effect of storage temperature on measured levels of HDL apolipoprotein C-III (apoC-III) and apoE. Plasma samples (n = 5) were stored for 2 or 6 days at 4°C or for 2 weeks at −20°C or −80°C. HDLs were separated by precipitation. Results are shown for individual samples. Horizontal bars represent mean values. Average increase in HDL apoC-III or apoE concentration at given time points is shown in each panel as a percentage.

served in HDL apoC-III and apoE levels after 2 or 6 days of storage.

In a previous study carried out in our laboratory (23), we determined the plasma lipoprotein distribution of apoC-III in freshly isolated plasma from normolipidemic (NL, n = 10) and hyperlipidemic (n = 30) subjects. Of the hyperlipidemic subjects, one-third (n = 10) were hypertriglyceridemic (HTG), one-third (n = 10) were hypercholesterolemic (HC), and one-third (n = 10) were both HTG and HC. After FPLC separation of plasma lipoproteins, mean (±SE) HDL apoC-III concentrations were 6.9 ± 0.6, 7.6 ± 0.7, 7.4 ± 1.0, and 7.2 ± 1.0 mg/dl in the NL, HC, HTG and HTG/HC groups, respectively (23). In percentage terms, HDL apoC-III represented 54 ± 5%, 46 ± 4%, 19 ± 3%, and 23 ± 3% of total plasma apoC-III, respectively. These results are shown graphically in **Fig. 4** relative to plasma TG concentrations. Data for NL and HTG subjects are shown with filled circles and data for HC and HC/HTG subjects are shown with open circles. An inverse relationship clearly exists between the relative amount of plasma apoC-III in HDL and the concentration of total TG, as described by others (27–29). From these data, it can be estimated that individuals with a plasma TG concentration of 1.5 mmol/l (133 mg/dl) tend to have 40% of total plasma apoC-III in HDL, while those with a plasma TG concentration of 2.0 mmol/l (177 mg/dl) tend to have ∼30–35% of total plasma apoC-III in HDL. In the present study, freshly isolated samples with a mean TG concentration of 2.3 ± 0.5 mmol/l (204 mg/dl) had 35 ± 5% of total plasma apoC-III in HDL-HMP and 38 ± 4% of total plasma apoC-III in HDL-FPLC. These results can be compared with those that have been published for HDL isolated from frozen plasma. For example, in 162 patients

at baseline in the Cholesterol-Lowering Atherosclerosis Study having a mean plasma TG of 153 ± 7 mg/dl (1.73 mmol/l), the ratio of HDL to non-HDL apoC-III was 1.4 ± 0.1 (15), equivalent to 58% of total plasma apoC-III in HDL. In patients (n = 220) at baseline in the Monitored Atherosclerosis Regression Study having a mean plasma TG of 160 ± 5 mg/dl (1.8 mmol/l), the ratio of HDL to non-HDL apoC-III was 1.7 ± 0.1 (16), equivalent to 63% of total plasma apoC-III in HDL. In control subjects (n = 370) from the Cholesterol and Recurrent Events trial, HDL apoC-III measured in frozen samples accounted for 46% of total plasma apoC-III (18). A greater proportion of plasma apoC-III is therefore recovered in HDL when frozen samples have been analyzed in published studies. This is consistent with the present results demonstrating that HDL apoC-III levels are overestimated by up to 50% if determined on HDL isolated from frozen samples. HDL apoE levels are also overestimated, and this overestimation is greater than that for HDL apoC-III, independent of the method used for isolating HDL (Tables 1 and 2).

In some studies, measurement of apoC-III and/or apoE in apoB- and non-apoB-containing lipoproteins has been carried out using samples that have not been previously frozen. For example, in the Etude Cas Temoins sur 'Infarctus du Myocarde study (14), apoC-III and apoE concentrations were determined within 6 days of blood collection. Apolipoproteins were measured in whole plasma and in plasma that was immunoprecipitated with a specific anti-apoB antiserum to separate lipoproteins devoid of apoB (i.e., HDL). In myocardial infarction survivor patients from France with a mean (±SD) TG of 175 ± 82 mg/dl, 64.5% of plasma apoC-III was measured as HDL apoC-III. In myocardial infarction patients from Northern Ireland with a mean (±SD) TG of 199 ± 88 mg/dl, 54.8% of plasma apoC-III was measured as HDL apoC-III. (As mentioned before, with freshly isolated plasma samples, we found 35 ± 5% of plasma apoC-III in HDL-HMP and 38 ± 4% in HDL-FPLC.) For apoE, 68% was isolated as HDL apoE in French patients and 61% in Irish patients (14). In the present study, freshly isolated samples with a mean TG concentration of 2.3 ± 0.5 mmol/l (204 mg/dl) had 25 ± 3% of total plasma apoE in HDL-HMP and 31 ± 4% of total plasma apoE in HDL-FPLC. These results suggest that both HDL apoC-III and HDL apoE levels have been overestimated (and apoB-containing lipoprotein apoC-III and apoE levels have been underestimated), even in those studies that have analyzed unfrozen samples. This is consistent with the present data demonstrating significantly increased HDL apoC-III and HDL apoE levels in unfrozen samples stored at 4°C for 2 or 6 days. After 6 days of storage at 4°C, HDL apoC-III levels had increased by 35% and HDL apoE levels had increased by 51%, independent of any change in HDL cholesterol (Fig. 3).

In all of the aforementioned studies, different methods have been used to separate HDL from apoB-containing lipoproteins (i.e., immunoprecipitation, immunoaffinity chromatography, size-exclusion chromatography, and polyanionic precipitation). It is difficult to identify which of these methods is ideal, inasmuch as each has advan-

Fig. 4. Relationship between HDL apoC-III (expressed as a percentage of total plasma apoC-III) and plasma TG concentration. Data are taken from reference 23. HDLs were separated by heparin-manganese precipitation from freshly isolated plasma samples. Results for 20 subjects with normal LDL cholesterol levels (<3.4 mmol/l) are shown with filled circles; those for 20 subjects with elevated LDL cholesterol levels (>3.4 mmol/l) are shown with open circles.

tages and disadvantages in terms of accuracy, reproducibility, and ease of measurement. Accurate determination of HDL apoE is particularly problematic, because apoE is readily displaced from lipoprotein particles and some apoE-rich HDL can be precipitated by heparin-Mn2$^+$ (30–33). The present results demonstrate that apoE is also particularly susceptible to time- and temperature-dependent lipoprotein redistribution in stored samples, i.e., in all our experiments, HDL apoE was affected to a greater extent than HDL apoC-III (Tables 1–3). It is probably for these reasons that contradictory results have been presented regarding HDL levels of apoE and risk of CAD (14, 17, 18). It is also for these reasons that apoE, despite pronounced effects on lipoproteins and atherosclerosis, cannot be considered an appropriate biochemical marker for determining risk of atherosclerosis.

The mechanism whereby storage causes redistribution of apoC-III and apoE from TRL and other apoB-containing lipoproteins to HDL has not been clearly defined by the present study. One possibility is that HDLs are modified during storage, perhaps through the activity of phospholipid transfer protein or cholesteryl ester transfer protein. We did not, however, observe large changes in HDL TG or cholesterol levels of samples stored at −20°C, which argues against this explanation. A second possibility is that time, freezing, and/or thawing may cause larger TRLs to fragment, resulting in transfer of apoC-III and apoE to HDL. This explanation is also unlikely, however, because no decrease was observed in levels of TG or cholesterol in TRL isolated from samples stored at −20°C. TG and cholesterol levels were actually higher in TRL from frozen samples (10 ± 2% and 72 ± 16%, respectively). Inspection of the lipid profiles shown in Figs. 1 and 2 suggests that this may have been at the expense of lipid in intermediate-sized remnant particles or large LDLs. Transfer of lipid from these lipoproteins or aggregation of smaller remnant particles to form larger TRLs may, therefore, cause displacement of apoC-III and apoE from the surface of these lipoproteins, with subsequent transfer to HDL. Evidently, this occurs to a greater extent in those samples that have higher TG levels and more apoC-III and apoE in TRL (Table 3). An increase in HDL apolipoprotein levels also occurs in samples frozen at −80°C (Fig. 3) and cannot be prevented by the addition of a stabilizing agent such as sucrose or by changing conditions of thawing. These results indicate that separation of lipoproteins needs to be carried out immediately after plasma isolation if accurate and physiological levels of apoC-III and apoE in different lipoprotein fractions are to be determined.

In conclusion, the present study has clearly demonstrated that storage of human plasma samples leads to alterations in the lipoprotein distribution of apoC-III and apoE. Although we have not been able to clearly define the mechanism responsible for the redistribution of apoC-III and apoE, it is clear that previous studies using frozen stored samples to measure lipoprotein apoC-III and apoE levels need to be interpreted with caution. Furthermore, our data suggest that any future studies relating lipoprotein levels of apoC-III and/or apoE to the incidence or prevalence of CAD need to be carried out on freshly isolated samples, or alternatively, lipoprotein fractions should be separated from fresh samples and then frozen for subsequent analysis. ∎

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