

# Modulation of Lipoprotein Lipase Activity by Apolipoproteins

## Effect of Apolipoprotein C-III

Chi-Sun Wang, Walter J. McConathy, Hans U. Kloer, and Petar Alaupovic

Laboratory of Lipid and Lipoprotein Studies and Oklahoma Lipid Research Clinic, Oklahoma Medical Research Foundation and Department of Medicine, Oklahoma University Health Sciences Center, Oklahoma City, Oklahoma 73104

### Abstract

From a total of 22 hypertriglyceridemic subjects tested, 14 subjects were selected on the basis of normal postheparin plasma lipoprotein lipase (LPL) levels and the presence of LPL inhibitory activity in their fasting plasma. The inhibitory activity was detected in both the lipoprotein fraction ( $d < 1.25$  g/ml) and the lipoprotein-deficient fraction ( $d > 1.25$  g/ml). Correlational analyses of LPL inhibitory activity and apolipoprotein levels present in the lipoprotein fraction ( $d < 1.25$  g/ml) indicated that only apolipoprotein C-III (ApoC-III) was significantly correlated ( $r = 0.602$ ,  $P < 0.05$ ) with the inhibition activity of the lipoprotein fraction. Furthermore, it was found that LPL-inhibitory activities of the plasma lipoprotein fraction and lipoprotein-deficient fraction were also correlated ( $r = 0.745$ ,  $P < 0.005$ ), though the activity in the lipoprotein-deficient plasma was not related to the ApoC-III or apolipoprotein E levels. Additional correlational analyses indicated that the LPL levels in the postheparin plasma of these subjects were inversely related to the levels of plasma apolipoproteins C-II, C-III, and E. To explain some of these observations, we directly examined the *in vitro* effect of ApoC-III on LPL activity. The addition of ApoC-III-2 resulted in a decreased rate of lipolysis of human very low density lipoproteins by LPL. Kinetic analyses indicated that ApoC-III-2 was a noncompetitive inhibitor of LPL suggesting a direct interaction of the inhibitor with LPL. Results of these studies suggest that ApoC-III may represent a physiologic modulator of LPL activity levels and that the incidence of LPL inhibitory activity in the plasma of hypertriglyceridemic subjects is more common than previously recognized.

### Introduction

One of the major functions of plasma lipoproteins is to transport and distribute triglycerides (TG)<sup>1</sup> throughout the organism. An important means of regulating the delivery of TGs to various tissues is through a rate-limiting hydrolysis step catalyzed by lipoprotein lipase (LPL) (1-4). There have

been numerous studies investigating the effect of LPL levels on this process. However, the possible role of apolipoproteins other than apolipoprotein C-II in modulating the LPL activity and controlling the flux of TGs through the lipid transport system has received limited attention, although several investigators have shown that some apolipoproteins inhibit LPL activity (5-10).

Both an increased influx of TG-rich lipoproteins and a decreased efflux of TG from the plasma compartment have been identified as pathophysiologic mechanisms leading to hypertriglyceridemia. Although overproduction of very low density lipoproteins (VLDL) is the apparent reason for the TG elevation in mild type IV hyperlipoproteinemia (11-13) and absence of LPL for the increased TG levels in type I hyperlipoproteinemia (14, 15), it is possible that other plasma factors such as apolipoproteins might also affect the TG levels in some types of hyperlipoproteinemia. In these cases, an increased plasma concentration of potential inhibitors of LPL may lead to reduced TG elimination despite adequate concentrations of enzyme protein at the surface of the capillary endothelium.

To further delineate the possible role of apolipoproteins in modulating the postheparin plasma LPL, we studied a group of hypertriglyceridemic (HT) subjects who had normal plasma postheparin LPL activity, but also possessed in their plasma a demonstrable inhibitory activity against LPL. We measured and correlated the LPL inhibitory activity with the concentrations of apolipoproteins A-I, A-II, B, C-II, C-III, D, and E. Only the plasma apolipoprotein C-III (ApoC-III) levels correlated positively with the LPL inhibitory activity. A kinetic study of the inhibitory activity of ApoC-III with partially purified bovine milk LPL as the enzyme source indicated that ApoC-III is a noncompetitive inhibitor of LPL. Results suggest that ApoC-III may be an important physiologic regulator of LPL activity that plays an important role in the flux of TGs through the plasma compartment.

### Methods

**Materials.** Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Beef lung heparin solutions for use in human subjects were obtained from the Upjohn Co., Kalamazoo, MI. Pig intestinal heparin was obtained from ICN Pharmaceuticals, Inc., Plainview, NJ. Glycerol tri[1-<sup>14</sup>C]oleate with a specific activity of 55 Ci/mol was obtained from Amersham Corp., Arlington Heights, IL. Heparin-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) was prepared as described previously (8).

**Hypertriglyceridemic subjects.** In that the purpose of this study was to define the modulating effect of apolipoproteins on the plasma LPL activity in hypertriglyceridemia, the selection of HT patients was based on normal levels of postheparin plasma LPL activity and the presence of an inhibitor(s) of LPL activity. Secondary causes of hyperlipoproteinemia were excluded by appropriate clinical and laboratory tests

Address correspondence to Dr. Wang, Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104.

Received for publication 27 February 1984 and in revised form 25 October 1984.

1. Abbreviations used in this paper: ApoC-III, apolipoprotein C-III; HT, hypertriglyceridemic; H-TGL, hepatic triglyceride lipase; LPL, lipoprotein lipase; TG, triglyceride.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/85/02/0384/07 \$1.00

Volume 75, February 1985, 384-390

and none of the patients were taking drugs known to affect plasma lipids. 22 male subjects (57±7 yr, range 42–66 yr) with fasting plasma triglyceride levels higher than 200 mg/dl were screened for their postheparin plasma LPL activity levels. Only one of these patients had a plasma LPL activity (4.6 U/ml) <2 standard deviations (SD) from the mean value for normolipidemic men (8). This subject was excluded from further studies. To identify HT subjects who had an inhibitor(s) of LPL activity, the inhibitory effect of their plasma on the lipolytic activity of postheparin plasma of a normolipidemic subject was determined according to a previously described procedure (8). This screening procedure indicated that plasma from seven patients caused <10% inhibition of the postheparin plasma lipolytic activity from a normal subject. These seven patients were also excluded from the study. The remaining 14 HT patients were then studied for the possible role of apolipoproteins in affecting their plasma LPL activity. Based on the criteria outlined by the Lipid Research Clinics (16), two of these patients were categorized as phenotype IIb (patients nos. 3 and 5, Table I), one as phenotype V (patient no. 11, Table I), and the remaining 11 patients as phenotype IV. For simplicity, we refer to this group of 14 subjects as HT patients.

**Measurement of postheparin plasma lipolytic activities.** Subjects were injected with 100 IU of heparin per kg of body wt up to the limit of 10,000 IU of heparin as maximum total dose. Blood was drawn 30 min after this injection, and the tubes were immediately placed on ice. Postheparin plasma, separated by centrifugation at 4°C, was stored at –20°C.

The determination of LPL and hepatic triglyceride lipase (H-TGL) was performed as described previously using a heparin-Sepharose 4B column chromatography procedure (8). 2 ml of postheparin plasma were mixed with 2 ml of 50 mM NH<sub>4</sub>OH-HCl buffer, pH 8.5, and applied to a heparin-Sepharose 4B column (0.7 × 2 cm). After applying the sample, the column was washed with 4 ml of 0.3 M NaCl

containing 50 mM NH<sub>4</sub>OH-HCl buffer; the LPL and H-TGL were eluted together with 4 ml of buffer containing 10 mg/ml heparin. H-TGL was measured directly from the heparin-eluted fraction in the absence of the activator, and the total activity was measured in the presence of 0.1 ml of human plasma as an activator according to the previously described assay (8). The difference between total activity and H-TGL activity represented the LPL activity.

**LPL inhibition assay.** Plasma from each of the HT patients was fractionated by ultracentrifugation into a lipoprotein (*d* < 1.25 g/ml) and lipoprotein-deficient (*d* > 1.25 g/ml) fraction. 2 ml of plasma were adjusted to *d* = 1.25 g/ml by the addition of solid KBr and made up to a final volume of 4 ml by the addition of an aqueous KBr solution, *d* = 1.25 g/ml. After centrifugation in a Ti50 rotor at 40,000 rpm for 24 h, the supernatant fraction (lipoprotein fraction) was removed by a tube-slicing technique. The infranatant fraction was washed once by the addition of the KBr solution, *d* = 1.25 g/ml, and centrifuged again at 40,000 rpm for 22 h. The upper fraction was removed and the lipoprotein-deficient fraction (*d* > 1.25 g/ml) was concentrated in a dialysis tubing (mol wt cutoff limit of 5,000) by polyethylene glycol to a small volume (~0.5 ml). Both the lipoprotein and lipoprotein-deficient fractions were dialyzed against 50 mM NH<sub>4</sub>OH-HCl buffer, pH 8.5, and adjusted to the initial starting plasma volume (2 ml).

Partially purified LPL was isolated from 2 ml of postheparin plasma from a normolipidemic subject by heparin-Sepharose 4B column chromatography according to a previously described procedure (8). In addition to 0.2 ml of the partially purified LPL fraction, Triton X-100-emulsified glycerol tri[1-<sup>14</sup>C]oleate (10 μmol/ml; 0.1 μCi) as substrate and bovine serum albumin (60 mg/ml in 50 mM NH<sub>4</sub>OH-HCl buffer) as fatty acid acceptor, the assay for measuring the inhibitory activity contained one of the following plasma combinations: (a) 0.1 ml of normolipidemic plasma and 0.1 ml of HT plasma, (b) 0.1 ml of normolipidemic plasma and 0.1 ml of HT lipoprotein fraction, and (c) 0.1 ml of normolipidemic plasma and 0.1 ml of HT

Table I. Plasma Levels of Lipids and Apolipoproteins and Plasma Postheparin LPL and H-TGL Activities of HT Patients

Subject	Phenotype	Total cholesterol	TG	Apolipoproteins							Lipolytic activities	
				A-I	A-II	B	C-II	C-III	D	E	LPL	H-TGL
				mg/dl	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl	U/ml	U/ml
1	IV	188	434	91	41	135	4.6	20.5	9.3	16.4	21.6	8.4
2	IV	232	257	171	66	213	5.5	16.4	9.5	9.8	21.5	13.3
3	IIb	274	253	126	60	176	4.3	17.5	11.6	15.8	18.7	4.9
4	IV	270	655	114	43	203	9.8	24.0	10.7	35.3	13.4	14.9
5	IIb	265	205	136	65	181	7.1	14.1	11.0	8.5	11.6	8.7
6	IV	218	497	118	47	107	6.4	19.4	16.0	23.5	18.0	7.8
7	IV	257	558	138	54	200	8.8	25.7	11.8	29.7	12.0	5.6
8	IV	269	402	148	66	166	5.9	24.8	9.7	20.4	14.3	11.5
9	IV	278	418	101	50	160	4.5	22.7	10.1	15.9	16.8	7.2
10	IV	245	491	111	44	164	6.8	21.2	11.9	24.3	13.8	10.1
11	V	259	1632	131	52	146	6.8	39.3	11.3	34.7	10.3	13.5
12	IV	304	792	137	60	154	4.9	14.3	10.3	13.0	22.4	8.8
13	IV	310	713	112	52	166	6.8	23.8	12.6	27.6	9.5	4.7
14	IV	310	690	114	65	178	7.8	30.4	12.0	21.7	12.0	6.1
Mean		262	571	125	55	168	6.4	22.4	11.3	21.2	15.4	9.0
(SD)		(35)	(354)	(21)	(9)	(28)	(1.6)	(6.7)	(1.7)	(8.5)	(4.4)	(3.3)
Normals*												
Mean		197	108	127	65	110	2.9	8.8	8.6	10.7	14.4	9.4
(SD)		(36)	(57)	(19)	(11)	(32)	(0.7)	(3.2)	(2.6)	(3.3)	(3.9)	(4.3)

\* Lipids and apolipoproteins for males (*n* = 122). LPL and H-TGL activity levels determined in 34 normolipidemic male subjects.

lipoprotein-deficient fraction. The final volume of the assay mixture was 1 ml and the assay was carried out as previously described (8).

With normolipidemic control plasma ([TG] < 150 mg/dl, total cholesterol [TC] < 250 mg/dl,  $n = 10$ ) using the same volume of plasma as used for hyperlipidemic sample, we could not demonstrate the presence of inhibitor activity in the assay system.

**Purification of human milk LPL and bovine milk LPL.** Human milk LPL was isolated from the acetone-diethyl ether powder of milk cream by heparin-Sepharose 4B affinity chromatography (17). The powder (40 mg) was dispersed with a pestle and solubilized in 4 ml of 50 mM  $\text{NH}_4\text{OH-HCl}$  buffer, pH 8.5, containing 0.1% Triton X-100 by stirring for 10 min at room temperature. The mixture was centrifuged at low speed for 2 min. The supernatant fraction was collected and applied to a small heparin-Sepharose 4B column ( $0.7 \times 2$  cm). After loading the sample, the column was washed first with 4 ml of 50 mM  $\text{NH}_4\text{OH-HCl}$  buffer, pH 8.5, containing 0.3 M NaCl and then 4 ml of the same buffer containing 0.72 M NaCl. After these washes, the LPL was eluted with 3 ml of 50 mM  $\text{NH}_4\text{OH-HCl}$  buffer containing heparin (10 mg/ml). The activity of LPL in the acetone-diethyl ether powder varied from batch to batch. For this study, the activity of the eluted LPL was in the range of 70–80 U/ml.

The purification of bovine milk LPL was performed as described by Posner et al. (18). Briefly, LPL was isolated from bovine skim milk by flotation with Intralipid (Cutter Laboratories, Berkeley, CA) followed by the preparation of acetone-diethyl ether powder. The powder (20 mg) was then dissolved in 4 ml of 0.1% Triton X-100 containing  $\text{NH}_4\text{OH-HCl}$  buffer and chromatographed as described for human milk LPL except that bovine milk LPL was eluted with 2 ml of 2 M NaCl containing buffer rather than with heparin solution. The eluted LPL was diluted 50-fold with ice-cold 50 mM  $\text{NH}_4\text{OH-HCl}$  buffer, pH 8.5, and used immediately for kinetic studies.

**Isolation of VLDL.** Human plasma samples were collected from normolipidemic and HT subjects who had fasted for 12–14 h. The plasma was centrifuged in a Ti60 rotor at plasma density at 140,000  $g$  for 24 h at 4°C. The supernatant fraction, removed by a tube-slicing technique, was adjusted to  $d = 1.006$  g/ml and centrifuged again under identical conditions. The VLDL isolated from normolipidemic subjects was dialyzed against 50 mM  $\text{NH}_4\text{OH-HCl}$  buffer, pH 8.5, and used as a substrate. Hypertriglyceridemic VLDL was used for the isolation of ApoC-III.

**Isolation of ApoC-III.** The apolipoprotein C-containing fraction was isolated from totally delipidized VLDL using the solubilization and DEAE-cellulose chromatography procedures previously described (19). Isolated ApoC-III-2 was subjected to flat-bed isoelectric focusing as described by Marcel et al. (20). After an additional focusing, the isolated ApoC-III-2 was chromatographed over a Sephadex G-25 column (Pharmacia Fine Chemicals) equilibrated with 2 M acetic acid. The isolated ApoC-III-2 was homogeneous as judged by analytical isoelectric focusing, and by basic and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21); it only reacted with antisera specific for ApoC-III. Amino acid analyses of this preparation were similar to those reported in the literature (22) and the protein content of ApoC-III-2 used in the kinetic studies was based on these analyses.

**Lipolysis of human VLDL with human milk LPL and the inhibitory effect of ApoC-III-2.** In a final volume of 6 ml, the lipolysis mixture contained VLDL (0.5 mg of TG/ml) without and with exogenously added ApoC-III-2 (0.1 mg/ml), bovine serum albumin (60 mg/ml), 50 mM  $\text{NH}_4\text{OH-HCl}$  buffer, pH 8.0, and human milk LPL (1 ml of heparin eluate). At this concentration of VLDL-TG (50 mg/dl), it can be estimated that the ApoC-III concentration is 2.5 mg/dl. At various time intervals, duplicate samples (0.5 ml) were removed and added to 4 ml of  $n$ -heptane-isopropanol (3:7, vol/vol), which contained 50  $\mu\text{g}$  of cholesterol butyrate as internal standard. After being acidified with 2.5 ml of 0.033 N  $\text{H}_2\text{SO}_4$ , the mixture was vortexed for 30 s, the organic phase containing the TGs and the internal standard was transferred to a 3-ml conical tube and the solvent was evaporated under nitrogen. The residue was redissolved in 100  $\mu\text{l}$  of  $n$ -hexane and

2- $\mu\text{l}$  aliquots were injected into the gas chromatograph for analysis of TGs (11).

**Kinetic study of the effect of ApoC-III-2 on bovine milk LPL activity.** The assay of LPL activity was performed in a final assay mixture (200  $\mu\text{l}$ ) in 50 mM  $\text{NH}_4\text{OH-HCl}$  buffer, pH 8.5, containing 60 mg/ml bovine serum albumin, 25 mM  $(\text{NH}_4)_2\text{SO}_4$ , and 2.5 mg/ml Triton X-100. The substrate triolein was adjusted to a specific activity of 0.1  $\mu\text{Ci}/\mu\text{mol}$ . The amounts of substrate, ApoC-III-2, and activator apolipoprotein C-II are shown in the legend of Fig. 3. The enzyme reaction was started by adding 20  $\mu\text{l}$  of purified bovine milk LPL. Triton X-100 was employed for emulsification (23) rather than phospholipid, in that phospholipid may also be a substrate for LPL (24). The incubation was performed at 37°C in a shaking water bath for 1 h. The reaction was terminated by adding 3.2 ml of chloroform-heptane-methanol (5:4:5.6, vol/vol/vol) and 1 ml of 0.2 N NaOH. After centrifugation, 1.2 ml of the top layer was mixed with 10 ml of Instagel (Packard Instrument Co., Inc., Downers Grove, IL) and the radioactivity was measured in a Packard liquid scintillation counter (Packard Instrument Co., Inc.). One unit of lipase activity is defined as 1  $\mu\text{mol}$  of fatty acid released per h at 37°C.

**Quantitative assay of apolipoproteins.** The preparation of antisera to apolipoproteins A-I, A-II, B, C-II, C-III, D, and E has been previously described (25, 26). The quantitative determination of apolipoproteins A-I, A-II, B, C-II, C-III, D, and E was performed by electroimmunoassays developed in this laboratory (25–30).

**Protein determination.** Protein content was determined by a modification (31) of Lowry's procedure (32). Bovine serum albumin was used as the standard.

**Analysis of results.** The  $K_m$  and  $V_{max}$  values derived from Lineweaver-Burk plots were based on least square linear regression analysis (33) assuming a constant variance of reaction velocities (computed with a CompuCorp 344 [Los Angeles, CA] statistician microcomputer). The measured initial rates had a coefficient of variation of <10%. For statistical analyses Student's  $t$  test was employed. Pearson correlational analyses of all measured variables were carried out using the Statistical Analysis System (SAS Institute, Inc., Raleigh, NC). Results are reported for correlations significant at the  $P < 0.05$  level.

## Results

**Plasma apolipoprotein profile and postheparin plasma lipolytic activities of HT patients.** Plasma or lipoprotein fraction ( $d < 1.25$  g/ml) from normolipidemic subjects ( $n = 10$ ) showed no inhibitory effect on the LPL activity in agreement with our previously reported results (8). In fact, the addition of the lipoprotein fraction resulted in a slight activation of the original LPL activity ( $2.6 \pm 2.3\%$ ). From 22 patients with TG levels greater than 200 mg/dl, one was eliminated from further studies because of a low postheparin plasma activity. Seven additional patients were eliminated because their plasma had <10% inhibitory effect on the postheparin plasma lipolytic activity of a normolipidemic subject. As expected, the HT patients whose plasma showed low or no inhibitory effect on lipolytic activity (<10%) had lower ( $P < 0.05$ ) fasting plasma TG levels ( $273 \pm 32$  mg/dl,  $\bar{x} \pm \text{SD}$ ) than those of the other HT subjects ( $571 \pm 354$  mg/dl). The former also had lower ApoC-III level ( $13.8 \pm 1.8$  mg/dl) than the latter ( $22.4 \pm 6.7$  mg/dl, Table I). However, the LPL levels of both groups were similar ( $14.8 \pm 3.8$  U/ml vs.  $14.4 \pm 3.9$  U/ml). These latter 14 patients were studied in more detail. The levels of plasma triglyceride, total cholesterol, apolipoproteins A-I, A-II, B, C-II, C-III, D, and E, and postheparin plasma LPL and H-TGL activities of the HT subjects are shown in Table I. As expected from the selection criteria, the HT patients had LPL levels similar to those of normolipidemic subjects. The mean H-TGL levels

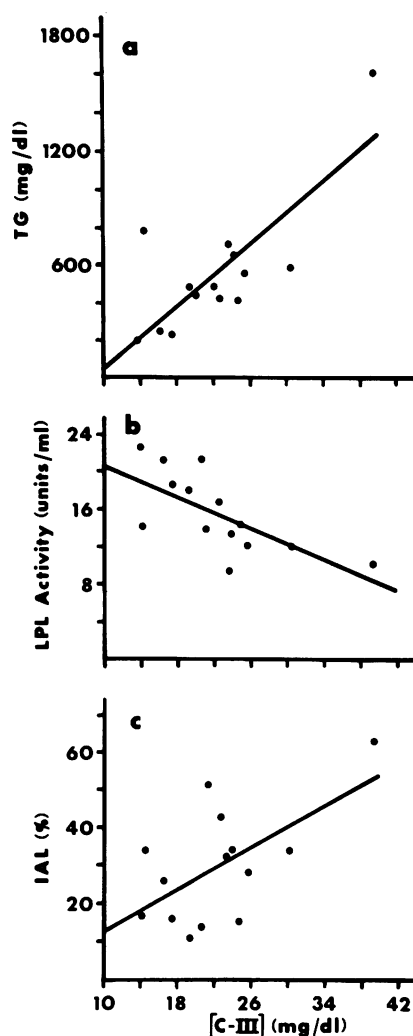
were also found to be similar to those of the normolipidemic population. Despite the higher TG levels, the plasma apolipoproteins A-I and A-II of HT patients were within the normal range. The mean concentrations of apolipoproteins B (53% higher), C-II (120%), C-III (150%), D (31%), and E (98%) were found to be higher than those of normolipidemic subjects (34). However, only apolipoproteins C-III ( $r = 0.780$ ,  $P < 0.005$ ; Fig. 1 a) and E ( $r = 0.653$ ,  $P < 0.05$ ) correlated significantly with fasting plasma TG levels in these HT subjects. The LPL activity was negatively correlated with plasma ApoC-II ( $r = -0.681$ ,  $P < 0.01$ ), ApoC-III ( $r = -0.614$ ,  $P < 0.05$ ; Fig. 1 b), and ApoE levels ( $r = -0.610$ ,  $P < 0.05$ ) whereas the ApoC-II/C-III ratio had no relationship to TG, LPL, or H-TGL levels.

**Effect of plasma lipoprotein and lipoprotein-deficient fractions from HT patients on LPL activity.** To establish the localization of the inhibitor(s) of postheparin plasma lipolytic

activity, the plasma samples from HT subjects were fractionated into a lipoprotein fraction ( $d < 1.25$  g/ml) and a lipoprotein-deficient fraction ( $d > 1.25$  g/ml). Results in Table II show that both fractions had inhibitory activity against normal human postheparin plasma LPL. The inhibitory activity of the lipoprotein fraction ( $d < 1.25$  g/ml) correlated significantly with plasma TG levels ( $r = 0.716$ ,  $P < 0.005$ ). It should be stressed, however, that the observed inhibition was not due to an isotope dilution effect by endogenous substrate, because the calculated isotope dilution effects were  $< 10\%$  with the exception of an HT patient whose plasma TG concentration was 1,600 mg/dl. Furthermore, the observed inhibition of LPL activity was greater than the isotope dilution effect. The inhibitory activity of the lipoprotein fraction also correlated positively with plasma ApoC-III levels ( $r = 0.602$ ,  $P < 0.05$ ; Fig. 1 c) but showed no correlation with plasma apolipoproteins A-I, A-II, B, C-II, D, and E.

Correlational analyses indicated that the LPL inhibitory activity of the lipoprotein-deficient plasma was positively related to the inhibitory activity of the lipoprotein fraction (Table II). To establish whether the inhibitory activity of lipoprotein-deficient fraction was possibly due to the presence of ApoC-III and/or ApoE, the concentrations of both apolipoproteins were determined in these fractions from all HT patients. There was no detectable ApoC-III in lipoprotein-deficient fractions from 11 HT patients, whereas the remaining 3 subjects only had trace amounts of ApoC-III ( $< 0.1$  mg/dl) in this fraction. In contrast, all lipoprotein-deficient fractions had small amounts of ApoE ( $1.8 \pm 1.4$  mg/dl; range, 0.1–4.6 mg/dl). However, there was no correlation between the ApoE levels and the LPL inhibitory activity of these lipoprotein-deficient fractions.

**Effect of ApoC-III-2 on VLDL lipolysis.** After identifying ApoC-III as a possible in vivo modulator of LPL activity from the correlational analyses, its in vitro effect on VLDL lipolysis



**Figure 1.** Relationships of plasma TG, postheparin plasma LPL, and LPL inhibitor activity of plasma lipoprotein fraction (IAL) with concentrations of plasma ApoC-III. (a) TG vs. ApoC-III ( $r = 0.780$ ,  $P = 0.0010$ ); (b) LPL vs. ApoC-III ( $r = -0.614$ ,  $P < 0.05$ ); (c) IAL vs. ApoC-III ( $r = 0.602$ ,  $P < 0.05$ ). Methodology for determination of each parameter is described in Methods. The lines were derived by least square linear regression analyses.

**Table II.** LPL Inhibitory Activity of Lipoprotein and Lipoprotein-deficient Fractions of HT Patients

Subject	Inhibitory activity of lipoprotein fraction	Inhibitory activity of lipoprotein-deficient fraction
	% Inhibition*	% Inhibition*
1	14	0
2	26	9
3	16	11
4	34	2
5	17	8
6	11	0
7	28	4
8	15	3
9	43	0
10	51	52
11	63	43
12	34	11
13	32	11
14	34	18

\* Inhibition of LPL activity isolated from a normolipidemic subject. See Methods for details.

was examined. The lipolysis of VLDL with human milk LPL in the presence and absence of exogenous ApoC-III-2 (10 mg/dl) indicated a retarded lipolysis of normolipidemic VLDL in the presence of added ApoC-III-2. The lipolysis of VLDL from one individual had pseudo-first order rate constants of  $0.029 \text{ min}^{-1}$  and  $0.067 \text{ min}^{-1}$  in the presence and absence of added ApoC-III-2, respectively (Fig. 2). Comparable values of  $0.020 \text{ min}^{-1}$  and  $0.055 \text{ min}^{-1}$  were found for the lipolysis of VLDL from another individual. Thus, on an average, in the presence of added ApoC-III-2, the VLDL degradation rate was decreased 2.5-fold.

**Mechanism of the ApoC-III-2 inhibition of LPL activity.** To determine the inhibition mechanism, we examined the effect of ApoC-III-2 on bovine milk LPL activity. As would be expected from the lack of sequence homology between apolipoproteins C-II and C-III-2, there was a noncompetitive inhibitory effect of ApoC-III-2 against ApoC-II as the variable in the Lineweaver-Burk plot (Fig. 3 a). ApoC-III-2 was also a noncompetitive inhibitor against triolein as a variable substrate in the presence of a saturating concentration of ApoC-II (Fig. 3 b).

## Discussion

This study was based on the hypothesis that the LPL activity in capillary endothelium is modulated by apolipoproteins and that increased levels of TGs in some HT patients may be due to an abnormal modulation of LPL activity. After blood is drawn, the concentrations of plasma apolipoproteins and their LPL inhibitory activity become fixed. Thus, the measurement of the LPL inhibitory activity and the quantitative determination of various apolipoproteins could lead to the identification of a specific apolipoprotein(s) responsible for this inhibition; the higher the concentration of such an inhibitory apolipoprotein, the greater the inhibition effect. Results of this study show that in HT plasma only the levels of ApoC-III correlated significantly with the LPL inhibitory activity. However, the correlation coefficient ( $r = 0.602$ ) was lower than what would be expected from a one-to-one relationship between inhibitor and inhibitory activity. This indicated that additional modulators of LPL activity might be present in the plasma lipoprotein fraction of HT patients as already suggested by other investigators (5-10).

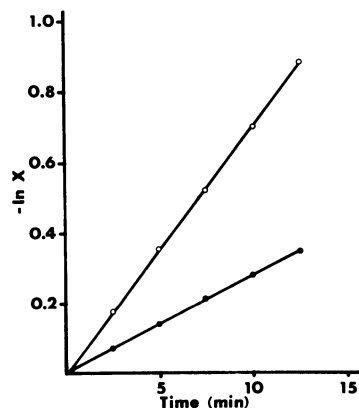


Figure 2. Effect of ApoC-III-2 on human plasma VLDL catalyzed by human milk LPL. Experimental details are described in Methods.  $X$  = fraction of VLDL-TG not hydrolyzed; (○), ApoC-III-2 = 0; (●), ApoC-III-2 = 10 mg/dl.

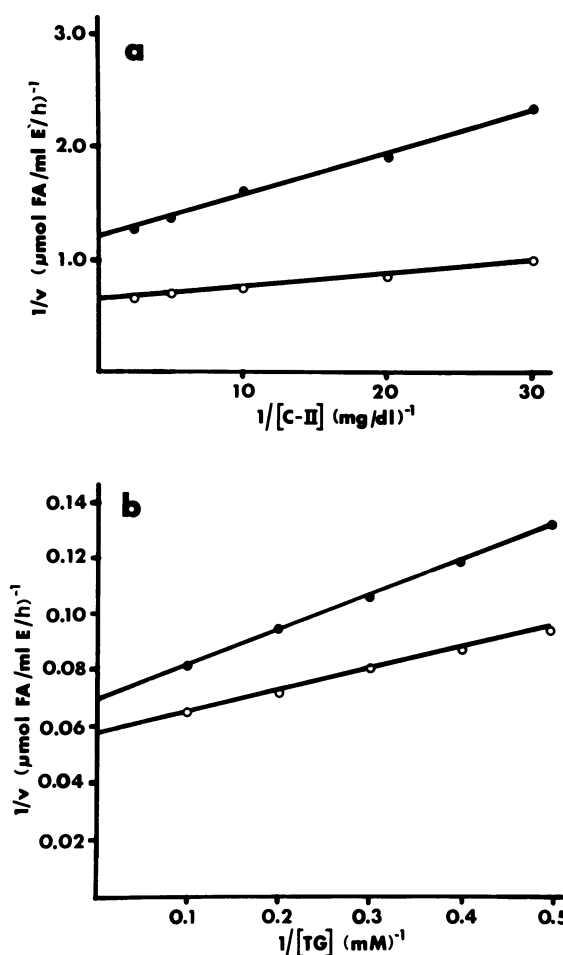


Figure 3. Effect of ApoC-III-2 on bovine milk LPL reaction kinetics. (a) Lineweaver-Burk plot with varying concentrations of ApoC-II. [Trioleoylglycerol] = 10 mM; (○), ApoC-III-2 = 0; (●), ApoC-III-2 = 3 mg/dl. (b) Lineweaver-Burk plot with varying concentrations of substrate trioleoylglycerol. [ApoC-II] = 1.0 mg/dl; (○), ApoC-III-2 = 0; (●), ApoC-III-2 = 3 mg/dl.

Because the apparent correlation between the levels of ApoC-III and LPL inhibitory activity could have been due to the presence of another, unidentified LPL inhibitor, it was necessary to establish in a direct fashion whether purified ApoC-III present at physiologic levels inhibits the LPL activity. Results of this study clearly identified ApoC-III as a physiologic modulator of LPL activity. The addition of ApoC-III-2 to VLDL reduced the rate of lipolysis of VLDL by purified human milk LPL. Further kinetic analyses showed that ApoC-III-2 represents a noncompetitive inhibitor against both ApoC-II and triolein in a reaction catalyzed by purified bovine milk LPL. The noncompetitive inhibitory effect of ApoC-III-2 against ApoC-II indicated that this effect was not due to its binding to the activator-binding site of LPL. In a previous study, Brown and Baginsky (9) also indicated that the inhibitory effect of ApoC-III could not be reversed by ApoC-II. The noncompetitive inhibitory effect of ApoC-III-2 against triolein indicated that the inhibition of LPL by ApoC-III-2 could only be partially reversed by increased substrate concentrations. Had the inhibitory effect of ApoC-III-2 been due to its binding to the substrate, and therefore, to a reduced effective substrate

concentration (35), such an inhibitory effect would have been reversed by a saturating amount of substrate. Furthermore, the Lineweaver-Burk plot would not be linear (35). Thus, these results suggest the presence of an ApoC-III-2 binding site on the LPL molecule.

Although the ApoC-II levels in HT subjects were increased, the ApoC-II/TG ratio (0.011) was still lower than that of normolipidemic subjects (0.027) (27). Because of the high-affinity binding of ApoC-II to LPL (36), it was considered that a saturating amount of this co-factor was available for activation of LPL and that ApoC-II did not represent the rate-limiting factor in the lipolysis of TG-rich lipoproteins (4).

Among other analyzed apolipoproteins (Tables I and II), ApoE showed the highest positive correlation with LPL inhibitory activity ( $r = 0.472$ ,  $P < 0.10$ ), though not at a significant level. In that ApoE has been implicated as a possible LPL inhibitor (8), its correlation with LPL inhibitory activity may have been masked by the more pronounced correlation between ApoC-III and the LPL activity (5, 6). However, additional studies are required to provide direct evidence for a physiologic role of ApoE in modulating LPL activity. It has been suggested that the ApoC-II/ApoC-III ratio may be an important plasma apolipoprotein factor affecting the normal removal of TG-rich lipoproteins (37, 38). The present study showed that such a ratio in HT plasma was not correlated with plasma LPL inhibitory activity.

An inhibitor of LPL activity was also found in the lipoprotein-deficient fraction of HT plasma. Correlational analyses suggested that this inhibition was not due to any of the apolipoproteins examined, but that it was possibly brought about by a nonapolipoprotein inhibitor similar, if not identical, to the nonlipoprotein inhibitor previously described in a case of familial hyperchylomicronemia (39). The LPL inhibitory activity of the plasma lipoprotein fraction correlated with the inhibitory activity of the lipoprotein-deficient fraction ( $r = 0.745$ ,  $P < 0.005$ ), and both were correlated with the concentration of plasma TG. These studies provide evidence that LPL inhibitory activity in HT plasma is more common than previously recognized.

To utilize enzyme activity as an indirect assessment of the enzyme concentration, it was necessary to remove the modulator(s) of LPL activity prior to the enzyme assay. This was accomplished by heparin-Sepharose column chromatography of postheparin plasma samples as previously described (8). Because normal levels of LPL activity were the main criterion for selecting HT patients, it was surprising to find a significant negative correlation between the LPL activity and levels of apolipoproteins C-II, C-III, and E. Thus, these apolipoproteins may be considered as potential apolipoprotein signals involved in the regulation of LPL activity level at the capillary endothelium. Alternatively, moderately decreased LPL levels at the capillary endothelium may lead to a gradual accumulation of VLDL with the accompanying increase in apolipoprotein levels that, in turn, may modulate the LPL activity.

Substrate availability in an enzymatic reaction is one of the important modes of metabolic regulation (40). In general, at substrate concentrations below the Michaelis constant ( $K_m$ ), the rate of enzyme reaction increases with increasing substrate concentration, and, at substrate concentrations greatly above  $K_m$ , the enzyme reaction operates at its saturating maximal rate. However, in the LPL catalyzed reaction, the enzyme

reaction seems to be more efficient at low concentrations of this natural substrate (VLDL). At high concentrations of substrate, as in this study, the enzyme reaction was less efficient because of the accompanying increases in the levels of ApoC-III and, possibly ApoE, and an increase in the inhibitory potential of the lipoprotein-deficient plasma.

Recent studies with recombinant DNA techniques have led to the suggestion that some cases of hypertriglyceridemia may be due to a single base pair substitution in the 3'-noncoding region of the ApoC-III gene (41, 42). Additional studies may provide a more precise insight into the mechanisms regulating the ApoC-III gene expression in normolipidemic subjects and patients with hypertriglyceridemia and clarify the role of inhibitors in the etiology and classification of HT states.

## Acknowledgments

The excellent technical assistance of Helen B. Bass, Deborah Downs, Tim Gross, and Jim Fesmire is appreciated.

These studies were supported, in part, by grants HD-14104 and HL-23181 and a Lipid Research Clinic Program contract (No. NO1-HV2-2932-L) from the National Institutes of Health and by the resources of the Oklahoma Medical Research Foundation.

## References

1. Robinson, D. S. 1970. The function of the plasma triglycerides in fatty acid transport. In *Comprehensive Biochemistry*, Vol. 18. M. Florkin and E. H. Stotz, editors. Elsevier Publishing Co., Amsterdam. 51-116.
2. Kompiang, I. P., A. Bensadoun, and M.-W. W. Yang. 1976. Effect of antilipoprotein lipase serum on plasma triglyceride removal. *J. Lipid Res.* 17:498-505.
3. Fielding, C. J., and R. J. Havel. 1977. Lipoprotein lipase. *Arch. Pathol. Lab. Med.* 101:225-229.
4. Nilsson-Ehle, P., A. S. Garfinkel, and M. C. Schotz. 1980. Lipolytic enzymes and plasma lipoprotein metabolism. *Annu. Rev. Biochem.* 49:667-693.
5. Havel, R. J., C. J. Fielding, T. Olivecrona, V. G. Shore, P. E. Fielding, and T. Egelrud. 1973. Cofactor activity of protein components of human very low density lipoproteins in the hydrolysis of triglyceride by lipoprotein lipase from different sources. *Biochemistry*. 12:1828-1833.
6. Twu, J.-S., A. S. Garfinkel, and M. C. Schotz. 1975. Rat heart lipoprotein lipase. *Atherosclerosis*. 22:463-472.
7. Bensadoun, A., C. Ehnholm, D. Steinberg, and W. V. Brown. 1974. Purification and characterization of lipoprotein lipase from pig adipose tissue. *J. Biol. Chem.* 249:2220-2227.
8. Wang, C.-S., H. B. Bass, D. Downs, and R. K. Whitmer. 1981. Modified heparin-Sepharose procedure for determination of plasma lipolytic activities of normolipidemic and hyperlipidemic subjects after injection of heparin. *Clin. Chem.* 27:663-668.
9. Brown, W. V., and M. L. Baginsky. 1972. Inhibition of lipoprotein lipase by an apolipoprotein of human very low density lipoprotein. *Biochem. Biophys. Res. Commun.* 46:375-382.
10. Jackson, R. L., F. Pattus, and G. de Haas. 1980. Mechanism of action of milk lipoprotein lipase at substrate interfaces: effects of apolipoproteins. *Biochemistry*. 19:373-378.
11. Grundy, S. M., H. Y. I. Mok, L. Zech, D. Steinberg, and M. Berman. 1979. Transport of very low density lipoprotein triglycerides in varying degrees of obesity and hypertriglyceridemia. *J. Clin. Invest.* 63:1274-1283.
12. Kissebah, A. H., S. Alfarsi, and P. W. Adams. 1981. Integrated regulation of very low density lipoprotein triglyceride and apolipoprotein-B kinetics in man: Normolipemic subjects, familial hypertriglyceridemia and familial combined hyperlipidemia. *Metab. Clin. Exp.* 30:856-868.
13. Chait, A., J. J. Albers, and J. D. Brunzell. 1980. Very low

- density lipoprotein overproduction in genetic forms of hypertriglyceridemia. *Eur. J. Clin. Invest.* 10:17-22.
14. Krauss, R. M., R. I. Levy, and D. S. Fredrickson. 1974. Selective measurement of two lipase activities in postheparin plasma from normal subjects and patients with hyperlipoproteinemia. *J. Clin. Invest.* 54:1107-1124.
  15. Nikkilä, E. A. 1983. Familial lipoprotein lipase deficiency and related disorders of chylomicron metabolism. In *The Metabolic Basis of Inherited Disease*. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, editors. McGraw-Hill Book Company, New York. 622-642.
  16. 1974. Manual of Laboratory Operations: Lipid and Lipoprotein Analysis, Vol. 1. Department of Health, Education, and Welfare Pub. No. (NIH) 75-628. 74-81.
  17. Wang, C.-S., D. Weiser, P. Alaupovic, and W. J. McConathy. 1982. Studies on the degradation of human very low density lipoproteins by human milk lipoprotein lipase. *Arch. Biochem. Biophys.* 214:26-34.
  18. Posner, I., C.-S. Wang, and W. J. McConathy. 1983. The comparative kinetics of soluble and heparin-Sepharose immobilized bovine lipoprotein lipase. *Arch. Biochem. Biophys.* 226:306-316.
  19. McConathy, W. J., C. Quiroga, and P. Alaupovic. 1972. Studies of the composition and structure of plasma lipoproteins. C- and N-terminal amino acids of C-I polypeptide ("R-Val") of human plasma apolipoprotein C. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 19:323-326.
  20. Marcell, Y. L., M. Bergseth, and A. C. Nestruck. 1979. Preparative isoelectric focusing of apolipoproteins C and E from human very low density lipoproteins. 1979. *Biochim. Biophys. Acta.* 573:175-183.
  21. Olofsson, S. O., W. J. McConathy, and P. Alaupovic. 1978. Isolation and partial characterization of a new acidic apolipoprotein (apolipoprotein F) from high density lipoproteins of human plasma. *Biochemistry.* 17:1032-1036.
  22. Brewer, Jr., H. B., R. Shulman, P. Herbert, R. Ronan, and K. Wehrly. 1974. The complete amino acid sequence of alanine apolipoprotein (apoC-III), an apolipoprotein from human plasma very low density lipoproteins. *J. Biol. Chem.* 249:4975-4984.
  23. Schotz, M. C., A. S. Garfinkel, R. J. Huebotter, and J. E. Stewart. 1970. A rapid assay for lipoprotein lipase. *J. Lipid Res.* 11:68-69.
  24. Muntz, H. G., N. Matsuoaka, and R. L. Jackson. 1979. Phospholipase activity of bovine milk lipoprotein lipase on phospholipid vesicles: influence of apolipoprotein C-II and C-III. *Biochem. Biophys. Res. Commun.* 90:15-21.
  25. Alaupovic, P., M. D. Curry, W. J. McConathy, and J. Fesmire. 1979. Electroimmunoassay of apolipoproteins occurring in high density lipoproteins of human plasma. In *Report of the High Density Lipoprotein Methodology Workshop*. Department of Health, Education, and Welfare Pub. No. (NIH) 79-1661. 227-240.
  26. Curry, M. D., A. Gustafson, P. Alaupovic, and W. J. McConathy. 1978. Electroimmunoassay, radioimmunoassay, and radial immunodiffusion assay evaluated for quantification of human apolipoprotein B. *Clin. Chem.* 24:280-286.
  27. Curry, M. D., W. J. McConathy, J. D. Fesmire, and P. Alaupovic. 1981. Quantitative determination of apolipoprotein C-I and C-II levels in human plasma by separate electroimmunoassays. *Clin. Chem.* 27:543-548.
  28. Curry, M. D., W. J. McConathy, J. D. Fesmire, and P. Alaupovic. 1980. Quantitative determination of human apolipoprotein C-III by electroimmunoassay. 1980. *Biochim. Biophys. Acta.* 617:503-513.
  29. Curry, M. D., W. J. McConathy, and P. Alaupovic. 1977. Quantitative determination of human apolipoprotein D by electroimmunoassay and radial immunodiffusion. *Biochim. Biophys. Acta.* 491:232-241.
  30. Curry, M. D., W. J. McConathy, P. Alaupovic, J. H. Ledford, and M. Popovic. 1976. Determination of human apolipoprotein E by electroimmunoassay. *Biochim. Biophys. Acta.* 439:413-425.
  31. Wang, C.-S., and R. L. Smith. 1975. Lowry determination of protein in the presence of Triton X-100. *Anal. Biochem.* 63:414-417.
  32. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
  33. Wang, C.-S. 1981. Human milk bile salt-activated lipase. Further characterization and kinetic studies. *J. Biol. Chem.* 256:10198-10202.
  34. Alaupovic, P., W. J. McConathy, and J. Fesmire. 1983. Apolipoprotein profiles of dyslipoproteinemic states. In *Proceedings of the Workshop on Apolipoprotein Quantification*. K. Lippel, editor. National Institutes of Health Pub. No. 83-1266. 27-53.
  35. Segel, I. H. 1975. *Enzyme Kinetics*. John Wiley & Sons, New York. 203-206.
  36. Posner, I., C.-S. Wang, and W. J. McConathy. 1983. Kinetics of bovine milk lipoprotein lipase and the mechanism of enzyme activation by apolipoprotein C-II. *Biochemistry.* 22:4041-4047.
  37. Carlson, L. A., and D. Ballantyne. 1976. Changing relative proportions of apolipoprotein C-II and C-III of very low density lipoproteins in hypertriglyceridemia. *Atherosclerosis.* 23:563-568.
  38. Erkelens, D. W., and J. A. J. Mocking. 1982. The C-II/C-III ratio of transferable apolipoprotein in primary and secondary hypertriglyceridemia. *Clin. Chim. Acta.* 121:59-65.
  39. Brunzell, J. D., N. E. Miller, P. Alaupovic, R. J. St. Hilaire, C.-S. Wang, D. L. Sarson, S. R. Bloom, and B. Lewis. 1983. Familial chylomicronemia due to a circulating inhibitor of lipoprotein lipase activity. *J. Lipid Res.* 24:12-19.
  40. Newsholme, E. A., and C. Start. 1973. *Regulation in Metabolism*. John Wiley & Sons, New York. 19-20.
  41. Karathanasis, S. K., J. McPherson, V. I. Zannis, and J. L. Breslow. 1983. Linkage of human apolipoproteins A-I and C-III genes. *Nature (Lond.)* 304:371-373.
  42. Rees, A., J. Stocks, C. C. Shoulders, D. J. Galton, and F. E. Baralle. 1983. DNA Polymorphism adjacent to human apoprotein A-I gene: relation to hypertriglyceridemia. *Lancet.* I:444-446.