STUDIES OF THE HYDROLYSES OF TRIACYLGLYCEROLS IN CHYLOMICRONS, VERY LOW- AND LOW-DENSITY LIPOPROTEINS BY C-I ACTIVATED LIPOPROTEIN LIPASE FROM POST-HEPARIN PLASMA OF NORMAL HUMAN SUBJECTS

Diana M. LEE, Devaki GANESAN and Helen B. BASS

Cardiovascular Research Program, Oklahoma Medical Research Foundation
825 N.E. 13th Street, Oklahoma City, OK 73104, USA

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1. Introduction

Post-heparin plasma (PHP) from human subjects has been shown to contain three distinct triacylglycerol lipases [1]. One of these is the protamine insensitive triacylglycerol lipase [2–4] and the other two are the protamine sensitive lipoprotein lipases (LPLs). The two LPLs are distinguishable on the basis of activation by C-I and C-II polypeptides of ApoC [1]. The C-I activated LPL, $LPL_{C\text{-}I}$, was found to be absent in the PHP of subjects with Type I hyperlipoproteinemia and was diminished to less than 40% in subjects with Type V hyperlipoproteinemia [5]. On the other hand, PHP from patients with Type III hyperlipoproteinemia was found to be deficient in concentration and enzyme activity of $LPL_{C\text{-}II}$ while no abnormality of $LPL_{C\text{-}I}$ was observed [5]. In these same studies it was found that the hydrolysis of CM-TG of chyle was only 10% of control values when CM were incubated with LPLs from PHP of Type I. Since Type I subjects have elevated CM but not VLDL, and Type III subjects have elevated LDL_1 and VLDL but not CM, these results suggested that two LPLs may have different specificities for TG carried in CM and VLDL. The purpose of this study was to investigate the hydrolysis of TG from plasma CM, VLDL and LDL_1 by highly purified $LPL_{C\text{-}I}$ isolated from PHP of normolipidemic subjects.

Abbreviations and definitions: PHP, post-heparin plasma; LPL, lipoprotein lipase; $LPL_{C\text{-}I}$, the C-I activated lipoprotein lipase; $LPL_{C\text{-}II}$, the C-II activated lipoprotein lipase; CM, chylomicrons, $S_f \geqslant 400$; VLDL, very low density lipoprotein, S_f 20-400; LDL_1, low density lipoproteins of S_f 12-20; DFP, diisopropyl fluorophosphate; TG, triacylglycerols; TC, total cholesterol; PL, phospholipids; FFA, free fatty acids; ApoB, Apolipoprotein B; ApoC, Apolipoprotein C, consisting of three non-identical polypeptides C-I, C-II and C-III; ApoD, Apolipoprotein D ('thin line' polypeptide); ApoE, Apolipoprotein E ('Arginine-rich' polypeptide).

2. Materials and methods

2.1. *Collection of post-heparin plasma*

Normolipidemic healthy volunteers of either sex (18–27 years) served as donors of PHP. After an overnight fast (14 h) 100 units/kg body wt (maximal dose was 10 000 units) heparin sodium (Upjohn Co., Kalamazoo, MI.) was administered intravenously and the blood drawn 30 min later into chilled plastic bags containing 50 ml of 4% sodium citrate. Plasma was separated by centrifugation at 4500 rev/min for 10 min at 4°C and stored at −15°C until further use.

2.2. *Isolation and purification of $LPL_{C\text{-}I}$*

The LPLs were isolated from three units (1320 ml) of PHP by a procedure utilizing enzyme–substrate complex formation followed by calcium phosphate gel chromatography [5]. The separation of $LPL_{C\text{-}I}$ from $LPL_{C\text{-}II}$ was carried out by gel filtration in Sephadex G-100 as previously described [1,5].

2.3. *Isolation of lipoprotein substrates*

CM were obtained from the plasma of normolipidemic healthy donors 4 h after the ingestion of a fatty meal comprising 1300 cal (900 cal from fat).

Three types of fatty meals were used: dairy cream, Lipomul® (primarily corn oil, Upjohn Co., Kalamazoo MI.) or safflower oil containing malt. Preservative, solution containing 5% EDTA, 5% thimerosal, 5% sodium azide and 0.1 M DFP was added to the plasmapheresis bag (1/100 of plasma volume). Polyallomer tubes containing 30 ml plasma were layered with 5 ml 0.85% NaCl solution containing 0.05% EDTA. CM were isolated by ultracentrifugation in a swinging bucket rotor SW 27 at 20 000 rev/min at 4°C for 30 min. CM floating to the top were collected and washed 5 times by recentrifugation after layering 5,10,10,25 and 25 ml 0.85% NaCl + 0.05% EDTA solution on top of 30,25,25,10 and 10 ml CM, respectively.

Outdated plasma (less than a month old) from non-fasted donors obtained from the blood bank was the source of VLDL and LDL_1 Normolipidemic plasma (serum TG < 170 mg/dl, TC < 260 mg/dl), and plasma from subjects who had normal TC but elevated TG (180–405 mg/dl of serum) were pooled separately. VLDL and LDL_1 were isolated either by ultracentrifugation according to the procedures described previously [6] or by phosphotungstate precipitation [7]. Both VLDL and LDL_1 were recentrifuged until free of albumin as tested by double diffusion with antibodies to albumin. Lipoproteins were then dialyzed against several changes of 0.15 M NaCl containing 0.05% EDTA, pH 7.0, prior to chemical analysis. DFP and NaN_3 were added back to the lipoproteins to be used as substrate to 1 mM and 0.05% respectively for preservation.

2.4. *Protein and lipid analyses*

A modified version of the Lowry method [8] as described by Gustafson et al. [9] was used to determine the protein content of lipoproteins. A micro adaptation of the Lowry method [8] was used to determine the protein content of enzyme. TG were determined by the Autoanalyzer [10]. The esterified and unesterified cholesterol and phospholipids were determined according to methods described previously [6].

2.5. *Polyacrylamide-gel electrophoresis*

The enzyme preparations and lipoprotein fractions were analyzed by electrophoresis in 7% polyacrylamide gel containing 8 M urea in basic [11] and acidic [12] buffers and the polypeptide bands were identified as described previously [5,13,14].

2.6. *Immunochemical method*

The immunochemical properties of lipoprotein fractions were studied by double diffusion with monospecific antibodies to all known apolipoproteins and their polypeptides as described previously [13].

2.7. *Assays of lipolytic activity*

Lipolytic activity of enzyme preparations was measured with [^{14}C]triolein emulsified in Triton X-100 as described previously [1]. Incubations were carried out at 37°C for 30 min or 1 h. Fatty acids (FA) were extracted by the method of Schotz et al. [15], and the radioactivity counted by Packard Liquid Scintillation Spectrometer.

2.8. *Hydrolyses of physiological substrates with purified $LPL_{C\text{-}I}$*

The physiological substrates were assayed in the following mixture (per ml): 3 mg of TG from CM, VLDL or LDL_1, 120 mg of defatted human albumin (Sigma Chemicals, St. Louis, MO.) and 25 μmoles of $(NH_4)_2SO_4$, pH 8.5. Lipoproteins in 0.15 M NaCl solution containing all preservative agents except thimerosal were concentrated to 10–15 mg TG/ml prior to use. Either 0.2 ml of PHP or purified $LPL_{C\text{-}I}$ at a concentration of 2–3 μg per ml of assay mixture, unless otherwise stated, was added and the mixture was incubated at 37°C. The extraction of FFA from two 0.5 ml aliquots at specified time intervals and titration has been previously described [16].

3. Results and discussion

3.1. *Characterization of substrates*

3.1.1. Characterization of protein moieties of lipoprotein substrates

The immunochemical and electrophoretic studies demonstrated the presence of ApoB, C-I, C-II, C-III and ApoE in VLDL and LDL_1. ApoD was found only in VLDL and LDL_1 isolated from plasma of hyperglyceridemic subjects. CM from subjects consuming any of the three different types of fat meals contained all of these peptides. Immunodiffusion pattern showed that

in CM, ApoC was in association with ApoD and ApoE but not with ApoB. A-I, A-II and albumin were frequently found in CM preparations, but they were not in association with either ApoB or ApoC: ApoD: ApoE complex. After six recentrifugations, two CM preparations were found to be free of immunochemically detectable albumin, A-I and A-II. These studies indicated that albumin is not an integral component of CM. They also cast serious doubt as to the question whether A-I and A-II polypeptides are the intrinsic protein components of these lipoprotein particles [17].

3.1.2. Chemical composition of lipoprotein substrates

The percent chemical compositions of CM, VLDL and LDL_1 are shown in table 1. No significant difference was observed in the lipid composition of CM produced by different fat meals, although the FA composition of TG was distinct for each type of fatty meal. The CM-TG were rich in C_{16}, $C_{18\text{-}1}$ and $C_{18\text{-}2}$ FA for subjects taking dairy cream, Lipomul® and safflower oil, respectively. The most striking difference between the three density fractions was in the TG/CE ratio, i.e., TG/CE ratio decreases with increasing density of lipoproteins. There was no difference between VLDL isolated from normolipidemic and hypertriglyceridemic plasma. It is interesting to note that the chemical position of LDL_1 isolated from hypertriglyceridemic plasma. It is interesting to note that the chemical compo sition of LDL_1 isolated from hypertriglyceridemic normolipidemic plasma LDL_1).

3.2. *Hydrolyses of TG in CM, VLDL and LDL_1*

The results of assays with [^{14}C]triolein emulsion to which C-I and C-II were added as activators indicated that LPL eluted in the first fraction from Sephadex G-100 column was indeed C-I activated LPL ($LPL_{C\text{-}I}$ in Table 2). The hydrolysis rates of CM, VLDL, LDL_1 and triolein emulsion with or without the addition of C-I or C-II by either $LPL_{C\text{-}I}$ or PHP are shown in Table 2. The data in this table indicate that: (1) the CM are the preferred physiologic substrates for $LPL_{C\text{-}I}$: (2) the CM-TG, rich in C_{16} and $C_{18\text{-}1}$ FA were far better substrates for $LPL_{C\text{-}I}$ than those containing $C_{18\text{-}2}$: (3) the hydrolytic rates for CM-TG rich in C_{16} and $C_{18\text{-}1}$ FA were 100 times greater than those for TG in VLDL and LDL_1, and 10 times greater than those for artificial substrate, triolein and C-I; the hydrolytic rate for CM-TG rich in $C_{18\text{-}2}$ FA was 4–6-fold higher than those for VLDL and LDL_1, and was

Table 1
Percent chemical composition of chylomicrons, VLDL and LDL_1[a]

Lipoprotein preparations	Protein	Phospholipids	Cholesterol esters	Free cholesterol	Triacylglycerols	Free fatty acids
Chylomicrons (n=5)	N.I.	5.90 ± 2.04	3.45 ± 1.11	1.34 ± 0.43	88.68 ± 3.34	0.31 ± 0.31
VLDL (n=7)	9.37 ± 0.96	18.03 ± 2.60	12.80 ± 3.46	5.61 ± 0.90	55.35 ± 5.76	N.I.
(Normolipidemic)	N.I.	19.61 ± 2.83	13.92 ± 3.76	6.10 ± 0.98	60.21 ± 6.27	0.15 ± 0.65
VLDL (n=3)	9.23 ± 1.59	18.29 ± 3.10	10.58 ± 1.15	4.83 ± 1.52	57.07 ± 3.62	N.I.
(High plasma TG)	N.I.	20.03 ± 3.39	11.58 ± 1.26	5.29 ± 1.66	62.49 ± 3.96	0.61
LDL_1 (n=5)	17.97 ± 3.91	24.24 ± 1.23	22.71 ± 5.01	5.76 ± 1.77	29.49 ± 3.97	N.I.
(Normolipidemic)	N.I.	29.37 ± 1.49	27.51 ± 6.07	6.98 ± 2.14	35.73 ± 4.81	0.41 ± 0.23
LDL_1 (n=2)	12.78 ± 0.29	21.08 ± 1.07	32.73 ± 2.46	7.35 ± 2.36	26.11 ± 6.21	N.I.
(High plasma TG)	N.I.	24.02 ± 1.21	37.29 ± 2.80	8.37 ± 2.68	29.75 ± 7.07	0.55 ± 0.46

[a] Mean ± S.D.; n = number of experiments; N.I. = data not included in the calculation.

Table 2
Hydrolyses of natural and artificial triacylglycerol substrates by LPL_{C-I} and PHP[a]

Experiments	LPL_{C-I}						PHP			
	Chylo-microns	VLDL	LDL_1	Triolein + C-I	Triolein + C-II	Triolein	Chylo-microns	VLDL	LDL_1	Triolein
1[b]							4.3	–	–	18.8
2[b,c]	52.70 (20.51)	0.30 (0.11)	0.50 (0.19)	2.57 (1)	–	–	1.5	2.65	–	7.40
3[b,c,d]	4.5 (45.0)	0 (0)	0 (0)	0.10 (1)	0.0 (0)	0.0 (0)	4.62	2.37	2.47	19.92
4[e,f]	50.15 (19.29)	0.54 (0.21)	0.28 (0.11)	2.60 (1)	0.08 (0.03)	0.05 (0.02)	6.8	–	–	19.25
5[g,f]	5.78 (0.80)	1.15 (0.16)	0.90 (0.12)	7.25 (1)	0.0 (0)	0.0 (0)	9.75	5.00	4.62	11.50

[a] The rates were expressed as μmoles FFA/ml enzyme or PHP (equivalent to 5 ml assay mixture) measured at 30 min. Numbers in parentheses were the relative rates.
[b] Chylomicrons isolated from donors who had taken dairy cream.
[c] VLDL and LDL_1, isolated from normolipidimic donors.
[d] The LPL_{C-I} enzyme concentration was approximately one-tenth the usual concentration.
[e] Chylomicrons isolated from donors who had taken Lipomul®.
[f] VLDL and LDL_1 isolated from donors with elevated serum TG levels.
[g] Chylomicrons isolated from donors who had taken safflower oil.
Note: The PHP in each experiment was not necessarily the same parent PHP from which LPL_{C-I} was isolated. However, the substrates in each experiment were identical for the comparison between LPL_{C-I} and PHP.

0.8 times that of artificial substrate with C-I: (4) there was no difference in the hydrolytic rates between VLDL and LDL_1 isolated either from normolipidemic or hypertriglyceridemic plasma: and (5) LPL_{C-I} hydrolyzed CM-TG rich in C_{16} and C_{18-1} FA generally at a much higher rate than PHP, whereas the opposite was true for the CM-TG rich in C_{18-2}.

3.3. *Kinetic studies on the hydrolysis of physiological substrates by LPL_{C-I}*

The plots of log[S] vs time of incubation for Experiment 3 and 5 are shown in fig.1. Similar to the to the data shown in table 2, the hydrolyses of VLDL and LDL_1 were either zero or minimal. The hydrolyses of both CM preparations proceeded at a constant rate only during the initial 10 min. In some cases, the rate appeared to increase after 30 min. Sometimes the concentration of titratable FFA were higher than the expected FA content in TG after incubation for more than 60 min. This phenomenon was particularly frequent with TG of CM rich in polyunsaturated FA and also when the LPL_{C-I} activity was relatively high. It was thought that lipid peroxidation may have occurred during lengthy incubation (even in the presence of EDTA), and the oxidative products when extracted with Dole mixture, might behave like an acid. Therefore, antioxidant was added to these assay mixtures to prevent this phenomenon. Indeed, addition of 0.02% glutathione to the incubation mixture prevented the occurrence of excessive titratable FFA (fig.1).

3.4. *Inhibition studies*

Protamine sulfate and 1 N NaCl are the inhibitors of LPLs and PHP [1,5]. In the present study DFP, NaN_3, thimerosal, serum and $d > 1.23$ g/ml fraction of serum were also tested as potential inhibitors. NaN_3 (0.05%) increased PHP lipolytic activities 5%. This activating effect of NaN_3 was probably due to its

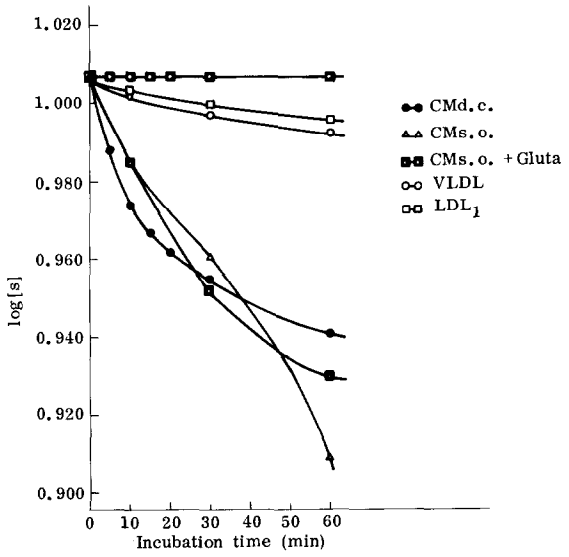

Fig.1. Kinetic behavior of LPL_{C-I} on lipoprotein substrates chylomicrons, VLDL and LDL_1. CM d.c., chylomicrons isolated from donors taking dairy cream; CM s.o., chylomicrons isolated from donors taking safflower oil; CM s.o. + Gluta., 0.02% Glutathione was added to the incubation mixture containing CM s.o. Composition of the incubation mixture for these assays, method of extraction and measurement of free fatty acids have been described under Materials and methods.

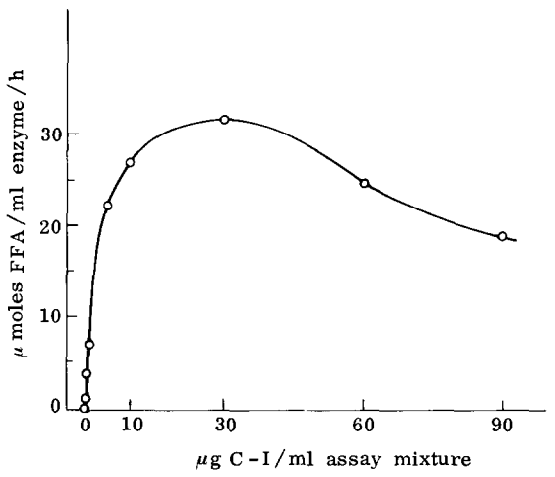

Fig.2. Activation of LPL_{C-I} by C-I polypeptide. Assay mixture contained 10 μmol [^{14}C]triolein emulsified with 45 μg Triton X-100, and other constituents as described previously [1].

stabilizing effect on the incubation mixture. At a concentration less than 0.5 mM DFP had no effect on PHP activities. Thimerosal was a potent inhibitor. Above 0.04%, it completely inhibited the PHP activities. Addition of normal serum and $d > 1.23$ g/ml fraction of serum (in 0.85% NaCl + 0.05% EDTA) at 0.2 ml/ml of assay mixture caused 22.6% and 88.8% inhibition of CM (polyunsaturated) hydrolysis by LPL_{C-I}.

At the present time we can only speculate about the reasons for the preferential hydrolysis of CM by LPL_{C-I}. Among the possible explanations including a preferential size of lipoprotein particles, a preferred TG/CE ratio, a preferred PL/TG ratio, we favor the last one. Data in fig.2 shows that the optimal activator concentration of C-I is 5–60 μg/10 μmol of TG in a triolein emulsion or a C-I/TG weight ratio of 0.6–0.68/100. Under identical conditions, 90 μg of C-I (corresponding to a 1/100 ratio for C-I/TG) activated LPL_{C-I} only 59% of the maximum obtained with 30 μg of C-I. Based on the assumption that C-I represents approximately 11% of CM protein [18], 14% of VLDL and 11% of LDL_1 protein [19] and TG content of these lipoprotein classes (table 1), it was calculated that the C-I/TG ratios for CM, VLDL and LDL_1 are 0.19/100, 2.3/100 and 5-7/100, respectively. These calculations show that only CM have an optimal C-I/TG ratio.

Conclusion

This is the first report of the hydrolyses of physiologic substrates by C-I activated LPL. Results show that CM are the preferred TG substrate. It is suggested that an optimal ratio of C-I/TG may be one of the factors for the substrate selectivity of LPL_{C-I}. CM derived from fat meals rich in C_{16} or C_{18-1}, TG-fatty acids are better substrates than those derived from exogenous TG rich in C_{18-2}. A non-dialyzable serum factor present in $d > 1.23$ g/ml inhibits LPL_{C-I}.

The data presented in this paper lends further strength to the previous report [5] that in Type I hyperlipoproteinemic subjects, the accumulation of CM is due to the deficiency of LPL_{C-I}. The sequential release of LPL_{C-I} and LPL_{C-II} in plasma after a fat meal [20] also suggests a significant physiologic role for LPL_{C-I} in the hydrolysis of CM.

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