

BBALIP 54120

Different hydrolytic efficiencies of adipose tissue lipoprotein lipase on very-low-density lipoprotein subfractions separated by heparin-Sepharose chromatography

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(Received 13 August 1992)

Key words: VLDL; Heparin-Sepharose; Apolipoprotein; Lipoprotein lipase; Triacylglycerol hydrolysis; Adipose tissue; (Human)

Human very-low-density lipoproteins (VLDL) were subfractionated by heparin-Sepharose chromatography into an unbound (A) and three bound (B, C and D) populations at increasing ionic strengths. Subfractions were characterized regarding their chemical composition and efficiency of triacylglycerol hydrolysis by rat adipose tissue LPL. The triacylglycerol content decreased, whereas the cholesterol and protein contents increased from subfractions A and B to subfraction D. VLDL-D showed the highest apo E/apo C ratio, though all the subfractions contained appreciable apo E. Appearance of VLDL-A resulted from exceeding the binding capacity of the column, since practically all its particles eluted at positions of bound VLDL under re-chromatography. Subfractions B, C and D stimulated LPL activity on emulsified tri[¹⁴C]oleoylglycerol to a similar extent, indicating that their apo C-II content was equally effective activating LPL. Incubation of tri[¹⁴C]oleoylglycerol labeled VLDL subfractions with fat pad pieces in the presence or absence of heparin resulted in greater hydrolysis and fatty acid uptake for VLDL-B and -C than for VLDL-D, a pattern observed over a wide range of LPL activities in the media. We conclude: (1) any VLDL particle can interact with heparin, which is consistent with the presence of apo E in all the subfractions, and (2) triacylglycerols in apo E-rich VLDL are less efficiently hydrolyzed by LPL than those in apo E-poor particles. We propose that richness in apo E impairs LPL action upon VLDL and decreases the rate of delivery of fatty acids to peripheral tissues.

Introduction

The plasma very-low-density lipoprotein fraction (VLDL; $d < 1.006$ kg/l) is a continuum of particles that are polydispersed and heterogeneous in size, composition [1,2] and the expression of certain superficial epitopes [3]. These characteristics modulate the particle's metabolic fate. VLDL mostly transports endogenously synthesized triacylglycerols from the liver to peripheral tissues in a process that requires the action

of lipoprotein lipase (LPL; EC 3.1.1.34), the rate-limiting enzyme for triacylglycerol hydrolysis (for a review see Ref. 4). Apolipoprotein (apo) E plays a central role in the catabolism of VLDL. Aside from being a ligand for membrane receptors, it has been postulated that functional apo E is required for the lipolytic conversion of VLDL into LDL to proceed normally [5]. Homozygotes for the mutant isoform E₂, whether normo [6] or hyperlipidemic (Type III) [7], show an impaired conversion pathway when compared to homozygotes for the commonest variant: E₃. Consistently, β -VLDL from Type III, E₂/E₂ patients is resistant to LPL-mediated hydrolysis and conversion into LDL-like particles *in vitro*, a phenomenon which is overcome by the addition of apo E₃, but not apo E₂ [8]. Moreover, in apo E-deficiency the synthesis of LDL is decreased and these particles are kinetically abnormal [9].

Heparin-Sepharose chromatography has been proven to be a suitable method for separating VLDL into subfractions of different apo E content [10–14]. Likewise, these subfractions have been shown to have a

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Abbreviations: VLDL, very-low-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPL, lipoprotein lipase; SDS, sodium dodecyl sulfate; KRB, Krebs-Ringer bicarbonate buffer.

distinct metabolic significance. LPL-mediated lipolysis *in vitro* [15,16] and kinetic studies in hyperlipidemic subjects [11,17] have provided evidences for a partial precursor-product relationship between apo E-poor and apo E-rich VLDL. The latter particles, in turn, are transformed into IDL [11]. On the other hand, apo E-rich VLDL is more effective than apo E-poor VLDL in competing with LDL for binding to the B/E-receptor of human fibroblasts [12,14]. However, the efficiency of LPL-mediated hydrolysis of these subfractions has not been addressed. The aim of the present work was to compare the LPL-mediated hydrolysis of the triacylglycerols carried by VLDL subfractions with a different apo E content. With this purpose human VLDL have been subfractionated by heparin-Sepharose chromatography and characterized for chemical composition. After being labeled *in vitro* with tri[¹⁴C]oleoylglycerol, these subfractions were incubated with rat epididymal fat pads to determine their hydrolysis by LPL.

Materials and Methods

VLDL preparation and subfractionation

Sera from normolipidemic donors who had fasted overnight were pooled and Na₂-EDTA (1 mg/ml), sodium azide (0.2 mg/ml) and aprotinin (Trasylol, 300 KIU/ml) were added as preservatives. VLDL ($d < 1.006$) was isolated by preparative ultracentrifugation at 40 000 rpm (145 500 $\times g$), at 10°C, for 18 h in a Beckman 50.2 Ti rotor (Beckman Instruments, Palo Alto, CA, USA). VLDL was recovered from the top by tube slicing and washed by an additional ultracentrifugation in the same conditions.

VLDL affinity chromatography was performed as described by Trezzi et al. [12] with minor modifications. The column (1.6 \times 20 cm) was packed with 40 ml of swollen heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) and equilibrated with 0.05 M NaCl/5 mM Tris-HCl (pH 7.4)/0.02% sodium azide containing 0.025 M MnCl₂ (buffer A). VLDL was dialyzed against 2.5 (\times 4) l of 0.05 M NaCl/5 mM Tris-HCl (pH 7.4)/0.02% sodium azide, 0.01% Na₂-EDTA, for 16 h, omitting Na₂-EDTA in the last two changes of medium. Immediately before loading the sample into the column, solid MnCl₂ was added to a final concentration of 0.025 M. VLDL was loaded in a volume of 10–15 ml (7–14 mg protein) at a flow rate of 30 ml/h and allowed to equilibrate for 2 h at 4°C. Elution was performed at a flow rate of 30 ml/h and 4°C, using a discontinuous salt gradient. The unretained fraction was eluted with buffer A and the retained particles were eluted stepwise by passing 5 mM Tris-HCl buffers (pH 7.4) containing 0.02% sodium azide and the following NaCl concentrations: 0.12 M (buffer B), 0.2 M (C), 0.5 M (D). The eluant was monitored at 280 nm.

The column fractions belonging to the same peak (designated A, B, C and D, respectively) were pooled, concentrated against sodium carboxymethyl-cellulose (Aquacide II, Calbiochem, San Diego, CA, USA) and dialyzed against 0.15 M NaCl, 0.01% Na₂-EDTA (pH 7.4). Subfractions prepared in this manner were shortly used for characterization and incubation. Triacylglycerol recovery after chromatography ranged between 70 and 90%, but the recovery degree did not significantly affect the proportion of the different VLDL subfractions. Re-chromatography of the subfractions will be commented in Results.

VLDL characterization

Protein content was determined by the method of Lowry et al. [18] using bovine serum albumin as standard; turbidity was removed by adding Triton X-100 to a final concentration of 0.1% [19]. Lipid composition was determined enzymatically. Triacylglycerols, choline-containing phospholipids and total cholesterol were measured using commercial kits from Menarini (Firenze, Italy) and unesterified cholesterol was measured using a kit from Boehringer-Mannheim (Mannheim, Germany). The cholesterol ester content was calculated by subtracting the unesterified cholesterol from the total cholesterol content. Mean diameter and mass of VLDL particles were estimated from their chemical composition as described by Redgrave and Carlson [20].

The apolipoprotein content of the VLDL subfractions was analyzed by SDS-polyacrylamide gradient gel electrophoresis according to the method of Laemmli [21]. Samples were previously delipidated with subsequent washes in acetone/ethanol (1:1, v/v) and diethyl ether. The pellet was redissolved with a Tris-HCl buffer (pH 6.8) containing 1% SDS (w/v) and 5% β -mercaptoethanol. Appropriate aliquots (approx. 65 μ g of protein) were loaded on slab gels (8 \times 10 cm, 0.75 mm thick) consisting of a 3.5% polyacrylamide (LKB) stacking gel (pH 6.8) and a 5–25% polyacrylamide gradient separating gel (pH 8.8), both containing 0.1% SDS. Electrophoresis was carried out at 50 V for 30 min and subsequently at 200 V until the tracking dye just left the gel. Gels were fixed and then stained with Coomassie Brilliant Blue R-250 and destained. The apolipoprotein bands were identified by means of appropriate molecular weight standards. The relative amounts of apolipoproteins were determined by densitometry at 560 nm.

The distribution of the apo E and apo C isoforms in VLDL was determined by isoelectric focussing in 7.5% polyacrylamide slab gels (7 \times 11.5 cm, 0.7 mm thick) containing 8 M urea and 2% ampholine (pH range 4–6.5; Pharmacia). Samples were delipidated as indicated above and the apolipoproteins were solubilized in a Tris-HCl buffer (pH 8.6) containing 8 M urea and

10 mM dithiothreitol. All the reagents were electrophoresis grade. The gels were prefocussed at 500 V for 30 min. Aliquots (approx. 80 μg of protein) of the samples were then applied to the gels and focussed at 500 V for 45 min and 2000 V for 150 min. Once the gels had been fixed they were stained with Coomassie Brilliant Blue R-250 and destained. The different isoforms were identified according to their isoelectric point [22] and quantified by scanning the gel at 560 nm.

VLDL labeling

VLDL was labeled *in vitro* with tri[1- ^{14}C]oleoylglycerol or tri[9,10(*n*)- ^3H]oleoylglycerol (Amersham, UK; specific activities = 59 mCi/mmol and 1 Ci/mmol, respectively) by incubation of unfractionated VLDL with radioactive triacylglycerol-coated Celite (Sigma, St Louis, MO, USA) according to the procedure of Breneman and Spector [23]. Labeled VLDL was dialyzed and subfractionated by heparin-Sepharose chromatography as described above. Radioactivity coeluted with the absorbance peaks, suggesting that radioactivity was specifically associated with the VLDL subfractions. VLDL-D regularly showed a 3 to 6-fold higher specific activity (dpm/ μg of triacylglycerol) than the rest of VLDL particles.

Incubation of the VLDL subfractions with rat adipose tissue

First, the ability of the VLDL subfractions to activate LPL was determined. The source of LPL consisted of the heparin-releasable enzyme from epididymal fat pads. For this fed male Wistar rats (approx. 200 g of body weight) were killed by cervical fracture and the epididymal fat pads were immediately excised and placed in Krebs-Ringer bicarbonate buffer (KRB) with a suitable salt content [24]. Fat pads from several animals were pooled and cut with scissors into small pieces (about 5 mg each). Fat pad pieces (2.6 g) were incubated in 85 ml of KRB containing 0.75 IU/ml of heparin, at 37°C for 25 min while gently shaking. After the incubation, the medium, containing LPL released from the tissue, was filtered by passing it through a nylon gauze. To determine LPL activity a tri[1- ^{14}C]oleoylglycerol/phosphatidyl-choline emulsion was used as substrate [25]. The reaction mixture contained 2 volumes of substrate emulsion and 3 volumes of 6% bovine serum albumin in 0.15 M NaCl/0.2 M Tris-HCl buffer (pH 8.2). The assay was performed by incubating 200 μl of the adipose tissue LPL solution and 100 μl of the reaction mixture (containing 624 nmol of trioleoylglycerol) in the presence or the absence of 500 μl (34 nmol of triacylglycerol) of whole VLDL or its subfractions in 0.15 M NaCl for 30 min at 37°C. Blank assay tubes without enzyme were also incubated to

make proper corrections. After 30 min of incubation 250 μl of the incubation mixture were transferred to another tube where the reaction was terminated by adding 3.5 ml of methanol/chloroform/heptane (141:125:100). ^{14}C -labeled unesterified fatty acids were extracted in the upper phase for counting after the addition of 1 ml of 0.1 M $\text{K}_2\text{CO}_3\text{-K}_3\text{BO}_3$ (pH 10.5) [26].

In another set of experiments, tri[1- ^{14}C]oleoylglycerol labeled VLDL subfractions were incubated in a final volume of 6 ml of KRB [24] containing 5 mM glucose and 1% bovine serum albumin in the presence or the absence (blank vials) of approx. 100 mg of fat pad pieces prepared as previously described. The adipose tissue weights corresponding to three independent experiments were: 103.7 ± 0.7 ($n = 16$), 102.3 ± 0.4 ($n = 20$) and 100.9 ± 0.5 ($n = 20$) (mean \pm S.E.) mg per vial. Half of the incubation vials were supplemented with 2.5 IU/ml of heparin [27]. This concentration of heparin produced maximum release of LPL activity to the media and maximum hydrolysis of VLDL-tri[1- ^{14}C]oleoylglycerol in our conditions. Incubations were performed for 120 min at 37°C in sealed glass vials gassed for 5 min with O_2/CO_2 (95:5) and shaken at 100 cycles/min. Incubations were terminated by transferring the vials to an ice bath and filtering the media through a nylon gauze to remove the tissue pieces. Immediately after this, 2 ml of the media were placed in 10 ml of chloroform/methanol (2:1) for lipid extraction [28] and fractionation in esterified and non esterified fatty acids [29]. Aliquots of these fractions were counted for radioactivity. The fat pad pieces were processed to determine the tissue uptake of [^{14}C]fatty acids. For this, the fat pad pieces were thoroughly washed with saline and placed in a scintillation vial where they were treated with 250 μl of Protosol (New England Nuclear, Bad Homburg, Germany). When the tissue was completely digested it was counted for radioactivity in 10 ml of a suitable scintillation cocktail (Normascint 11, Scharlau, Barcelona, Spain). Triacylglycerol hydrolysis (disappearance of esterified fatty acids), appearance of unesterified fatty acids in the media and tissue uptake of fatty acids were estimated from the proper dpm values and expressed as percentage of initial triacylglycerols by correcting for the specific activity (dpm/nmol of triacylglycerols) of the corresponding VLDL subfraction. An aliquot of the media was used to measure LPL activity as described [27].

Statistical analysis

Statistical comparisons were done using the Statgraphics version 5.0 (Statistical Graphics Corporation) on an IBM PS/2 computer. The analysis included Friedman two-way test by ranks and Wilcoxon test by ranks.

Results

Fig. 1 shows a representative profile of the heparin-Sepharose chromatography of VLDL from a pool of human sera when 11 to 14 mg of protein were loaded into the column. In these conditions four peaks, A, B, C and D in order of elution, appeared. The chemical composition and size of these particles are shown in Table I. VLDL-A and B had the highest triacylglycerol content and the lowest unesterified cholesterol, cholesterol ester and protein contents, whereas subfraction D showed opposite characteristics. In accordance, the estimated particle diameter progressively decreased from subfractions A and B to subfraction D. The apolipoprotein content of VLDL subfractions was analyzed by SDS gradient gel electrophoresis and subsequent densitometry. As shown in Fig. 2, Apo E was present in all the subfractions although, as expected from its high affinity to heparin, VLDL-D was the richest in this peptide, a fact made clearly manifest when the relative intensities of the apo E and apo C bands and the apo E/apo C ratio were considered (Fig. 2). However, despite the inability of VLDL-A to bind to heparin, its apo E content was higher than that corresponding to subfractions B and C (Fig. 2). To analyze the distribution of apo E and apo C isoforms, the VLDL subfractions were subjected to isoelectric focussing. There were no substantial differences in the distribution of the apo C-II and apo C-III isoforms between VLDL subfractions (Fig. 3). In agreement with the diverse source of the sera that were pooled to isolate VLDL, several apo E isoforms were identified in total VLDL, E₃ being the most abundant. Subfractions A and D displayed this same pattern; however, apo E was barely detectable in subfractions B and C (Fig. 3), which confirms their relatively low content in this apolipoprotein.

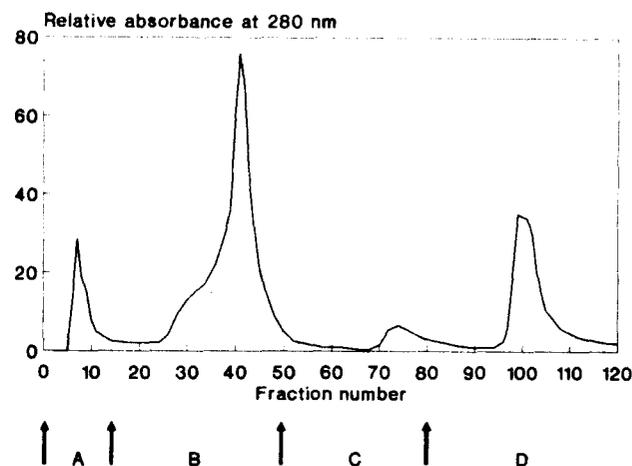


Fig. 1. A typical elution profile of VLDL from a heparin-Sepharose CL-6B column when 11–14 mg of protein were loaded. 11.6 mg of protein were applied and chromatography was performed as indicated in Materials and Methods. Fractions of 2 ml were collected. Arrows indicate the initiation of the different buffer steps (A, B, C and D).

To test whether subfraction A was the result of saturation of the column binding capacity different amounts of VLDL were subjected to heparin-Sepharose chromatography. When less than 11 mg of protein were loaded into the column no material was eluted with the first buffer (0.05 M NaCl/0.025 M MnCl₂) and only peaks B, C and D were obtained. Presence of peak A was exclusively apparent when 11 mg or more protein were loaded, and its proportion increased parallel to the amount of VLDL loaded (results not shown). To ascertain the origin of the particles in peak A, VLDL were labeled *in vitro* with tri[³H]oleoylglycerol, subfractionated in heparin-Sepharose columns and the resulting ³H-labeled VLDL-A, -B and -D were extensively dialyzed against 0.05 M

TABLE I

Percent lipid and protein composition of total VLDL and its subfractions

Values (mean ± S.E.) were obtained from four independent pools of sera as described in Materials and Methods. Particle diameter and mass were estimated from their chemical composition and partial specific volumes of the components [20]. Statistical comparisons between each pair of subfractions were performed using a Wilcoxon test by ranks. Differences are statistically significant ($P < 0.05$) when subfractions do not share any superscript letter. Friedman two-way analysis by ranks showed significant differences ($P < 0.05$) between subfractions for the cholesterol ester component after excluding VLDL-A from the analysis.

	VLDL				Total
	A	B	C	D	
Triacylglycerol	57.5 ± 2.3 ^a	57.8 ± 6.0 ^a	54.1 ± 5.3 ^{ab}	52.0 ± 3.6 ^b	57.7 ± 1.7
Cholesterol ester	4.7 ± 0.8 ^a	4.4 ± 1.0 ^a	5.2 ± 0.9 ^a	6.2 ± 0.9 ^b	5.9 ± 0.3
Unesterified cholesterol	6.0 ± 0.6 ^a	5.7 ± 1.0 ^{ab}	6.4 ± 0.9 ^{ab}	6.5 ± 0.5 ^b	5.7 ± 0.5
Phospholipid	18.2 ± 1.2 ^{ab}	18.2 ± 3.5 ^{ab}	18.0 ± 2.0 ^a	19.2 ± 1.6 ^b	17.8 ± 0.8
Protein	12.7 ± 2.1 ^a	13.8 ± 1.4 ^{ac}	16.4 ± 2.7 ^b	16.1 ± 1.7 ^{bc}	13.0 ± 1.1
Estimated diameter (nm)	35.8 ± 4.0 ^a	36.3 ± 7.1 ^{ab}	32.6 ± 5.3 ^{ab}	30.5 ± 3.2 ^b	35.6 ± 2.2
Estimated particle mass (10 ⁶ Dalton)	15.1 ± 4.4 ^a	18.1 ± 9.4 ^{ab}	12.6 ± 5.4 ^{ab}	9.5 ± 2.8 ^b	14.2 ± 2.4

As has been pointed out above, all the VLDL subfractions contained substantial amounts of apo C-II (Fig. 3), the physiological activator of LPL. To determine if these amounts were sufficient to activate LPL, the effect of adding a small amount (34 nmol triacylglycerol) of the VLDL subfractions as sources of apo C-II on the activity of LPL from rat adipose tissue was tested. A tri[¹⁴C]oleoylglycerol/phosphatidyl-choline emulsion was used as substrate. The addition of total VLDL produced a 2-fold increase in LPL activity versus the absence of an apo C-II source (from 6.25 to 12.15 pkat/ml). The stimulatory effect of each VLDL subfraction separately on LPL activity was similar to the effect of the total VLDL (12.13, 11.61 and 12.89 pkat/ml for subfractions B, C and D, respectively), which means that all the subfractions were equally effective in activating LPL.

To determine the efficiency of LPL hydrolysis of the triacylglycerols contained in the VLDL subfractions, tri[¹⁴C]oleoylglycerol labeled VLDL-B, -C and -D were incubated in the presence or the absence (blank incu-

TABLE II

Incubation of tri[¹⁴C]oleoylglycerol labeled VLDL subfractions in the presence of rat fat pad pieces

Results correspond to three independent experiments in which tri[¹⁴C]oleoylglycerol labeled VLDL subfractions at two different concentrations (I and II) were used. VLDL-triacylglycerol concentrations were: 8.5 and 25.4, 12.3 and 35.3 and 11.3 and 34.1 (I and II, respectively) nmol/ml for experiments 1, 2 and 3, respectively. At 2 h of incubation the disappearance of esterified fatty acids (hydrolysis), appearance of unesterified fatty acids in the media and uptake by the tissue were determined and expressed as percent of initial triacylglycerols by correcting for the specific activity (dpm/nmol of triacylglycerol) of the corresponding subfraction. LPL activity was measured in the media at the end of the incubation. Friedman two-way analysis by ranks showed significant differences between subfractions for hydrolysis ($P < 0.01$) and uptake ($P < 0.01$). Statistical comparisons between each pair of subfractions using Wilcoxon analysis by ranks showed the following significant differences ($P < 0.05$): Hydrolysis, B vs. D and C vs. D; Appearance of unesterified fatty acids, C vs. D; Uptake, B vs. D and C vs. D.

Exp. ^a	VLDL	% of initial triacylglycerols						LPL activity in media (pkat/100 mg of tissue)
		hydrolysis		fatty acids appearance		uptake		
		I	II	I	II	I	II	
1	B	55.5	58.3	18.3	30.5	26.7	22.4	216.1 ± 35.7 ^b
	C	50.8	53.4	12.3	25.3	23.4	17.7	
	D	24.0	27.1	6.1	14.6	13.2	11.1	
2	B	35.0	38.9	15.4	26.0	16.4	14.0	65.2 ± 11.0
	C	31.8	44.4	19.6	34.6	21.5	18.0	
	D	30.1	29.1	21.2	21.0	5.8	9.1	
3	B	19.0	18.6	17.8	12.7	13.6	9.2	18.6 ± 4.2
	C	9.7	11.8	6.5	8.8	4.2	8.0	
	D	2.0	5.7	1.0	5.2	1.6	3.8	

^a Expt. = Experiment;

^b means ± S.E. ($n = 8$).

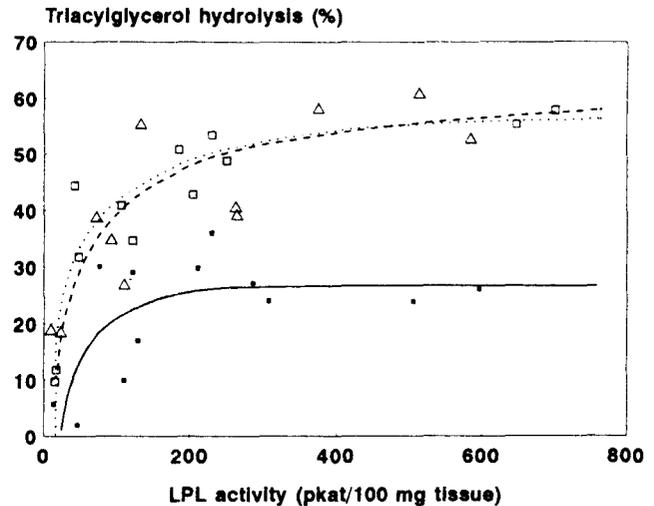


Fig. 5. Relationship between hydrolysis of triacylglycerols from the VLDL subfractions by rat fat pad pieces and LPL activity in the media. Results correspond to three independent experiments and include tri[¹⁴C]oleoylglycerol labeled VLDL concentrations I and II, both in the absence (Table II) and the presence of 2.5 IU/ml of heparin. Hydrolysis is expressed as percent of initial triacylglycerols. Addition of heparin resulted in the following mean LPL activities in the media: 583.8 ± 23.2 , 241.2 ± 8.3 and 112.5 ± 3.7 (\pm S.E., $n = 8$) pkat/100 mg tissue/2 h incubation for experiments 1, 2 and 3, respectively. Δ , VLDL-B; \square , VLDL-C; \blacksquare , VLDL-D. Friedman two-way analysis by ranks showed significant differences between subfractions for hydrolysis in the presence of heparin ($P < 0.05$) and when all data were considered ($P < 0.001$). Using Wilcoxon analysis by ranks the following differences between pairs of subfractions appeared: B vs. D ($P < 0.05$) and C vs. D ($P < 0.05$) in the presence of heparin; B vs. D ($P < 0.01$) and C vs. D ($P < 0.01$) when all data were considered.

bations) of rat fat pad pieces. The results from three different experiments carried out with independent pools of VLDL and adipose tissues are compiled in Table II. Triacylglycerol hydrolysis with subfractions B and C, as measured from the hydrolysis of tri[¹⁴C]oleoylglycerol, was consistently greater than with subfraction D. A certain proportion of the released [¹⁴C]fatty acids were taken up by the adipose tissue and the rest remained in the medium. The appearance of unesterified [¹⁴C]fatty acids and their uptake by the tissue preparations paralleled the hydrolysis of tri[¹⁴C]oleoylglycerol (Table II).

In order to maximally augment the availability of enzyme in the media, parallel incubations were performed in the presence of optimal amounts of heparin. Addition of heparin markedly enhanced LPL activity in the media (2.7, 3.7 and 6-fold on the average for experiments 1, 2 and 3, respectively) at the end of the incubation time. In order to analyze the effect of LPL activity, the data from the hydrolysis of VLDL subfractions were plotted versus LPL activity in the media, independently of whether samples were incubated in the absence or presence of heparin (Fig. 5). A hyperbolic relationship appeared in all cases between LPL

activity and triacylglycerol hydrolysis, but whereas no difference could be found between the kinetics of subfractions B and C a clear decreased maximal LPL efficiency to hydrolyze triacylglycerols was found in subfraction D. Similar relationships were obtained when plotting tissue uptake of released unesterified fatty acids versus LPL activity and the effects of the three labeled subfractions were compared (data not shown).

Discussion

In the present study VLDL subfractions with different affinities to heparin have been characterized regarding their chemical composition and the efficiency of LPL-mediated triacylglycerol hydrolysis. VLDL were subfractionated according to the procedure described by Trezzi et al. [12] to separate an unbound population (A) plus three subfractions (B, C and D) that were eluted by gradually increasing the ionic strength. The unbound particles we isolated are not a true subfraction as defined by a unique and reproducible affinity to heparin, but they do correspond to a population of particles from subfractions B, C and D that are not retained when the binding capacity of the column has been surpassed. Recently, Evans et al. [30] have reported that all the S_f 60–400 particles from Types IV and V hyperlipoproteinemic subjects can really bind to heparin in the presence of 0.05 M NaCl; now we have extended this concept to the whole VLDL population (S_f 20–400) from normolipidemic subjects. The fact that in our conditions practically all the VLDL are able to interact with heparin agrees with the presence of apo E in all the subfractions. Although apo B-100 [31] and apo E [32] both contain heparin binding sites, Fielding et al. [16] have shown that apo E, but not apo B-100, is the ligand that mediates binding of normal VLDL to heparin.

In agreement with previous reports [10–14], the VLDL that bound to heparin with the highest affinity, i.e., subfraction D, was not only the richest in apo E but also displayed the highest percentages of cholesterol and protein, the lowest percentage of triacylglycerols and the smallest size, whereas the VLDL that bound to heparin with the lowest affinity, i.e., subfraction B, displayed opposite characteristics. These features suggest that subfractions B, C and D represent VLDL at different stages of its metabolism, so that the extent of degradation increases from subfraction B to subfraction D. This view is also supported by our results showing that tri[14 C]oleoylglycerol contained in VLDL-B and -C is more efficiently hydrolyzed by LPL from rat adipose tissue than that contained in VLDL-D. Tissue uptake of triacylglycerol fatty acids paralleled the extent of triacylglycerol breakdown, with subfraction D being a poorer donor than subfractions B and

C. This agrees with the concept that the rate at which LPL acts on VLDL-triacylglycerols controls the cellular uptake of the products [27,33]. Thus, our results on VLDL hydrolysis and product utilization together with previous observations suggest that as the LPL-catalyzed lipolysis progresses the particle is enriched in apo E [15,16] and loses its ability to efficiently interact with the enzyme and, hence, to deliver triacylglycerol fatty acids to peripheral tissues. Apo E-rich VLDL particles are then preferentially driven to interact with hepatic receptors for clearance [12,14] or to the formation of $d > 1.006$ kg/l lipoproteins [11]. In connection with this last point, the possibility exists that apo E-rich VLDL is a better substrate for hepatic lipase. In vitro [34] and in vivo [35] studies have suggested that this lipase acts preferentially upon small, apo E-rich VLDL and IDL, whereas LPL does so upon large, triacylglycerol-rich particles.

The origin of the differences observed in triacylglycerol hydrolysis by LPL between VLDL subfractions must lie in their chemical and/or physical properties. Apo C-II did not seem to be limiting in any of the subfractions, since all of them contained this apolipoprotein and stimulated LPL activity to a similar extent. This is consistent with data suggesting that very little apo C-II is required to maximally activate LPL [36,37]. Even though the incubations were performed in the presence of equal amounts of triacylglycerols in all the subfractions, the absolute amount of triacylglycerols per particle may have influenced LPL activity. Recently, Saheki et al. [38] have reported that the parameter that best correlated with the LPL-mediated hydrolysis of whole VLDL in vitro is the ratio triacylglycerol/total or individual surface components. Theoretically, and assuming that the particles have enough apo C-II to fully activate LPL, the smaller the particle size the greater the number of particles that are needed for LPL to achieve a certain degree of hydrolysis. Although the consequences of this peculiarity are not known, we may speculate that the overall hydrolysis rate is impaired when LPL must shift from one particle to another with a high frequency. The percentual triacylglycerol content of the subfractions studied herein was not markedly different, but the reduction in particle size from VLDL-B to VLDL-D accounts for a considerable decrease in the number of triacylglycerol molecules (11 900, 7800 and 5600 molecules/particle for subfractions B, C and D, respectively, on average as estimated from their particle mass and composition in Table I). Thus, in order to achieve an equal amount of triacylglycerols in subfractions B, C and D, particles in approximate proportions of 1:1.5:2, respectively, are required. However, the observation that under conditions of excess LPL subfraction D still showed a lower degree of hydrolysis than subfractions B and C indicates that the proportion of VLDL particles versus

LPL molecules did not account for the different triacylglycerol hydrolysis rates between VLDL subfractions.

Probably, the compositional characteristic that most differentiated subfractions B and C from subfraction D was the apo E/apo C ratio. A role for apo E in the conversion of VLDL into LDL has been proposed [5], though the underlying mechanism is intriguing. Homozygosity for apo E₂ [6,7] and apo E-deficiency [9] are associated to a low LDL synthetic rate. Consistently, apo E₂/E₂ β -VLDL interacts deficiently with LPL, but addition of apo E₃ allows its conversion into LDL-like particles [8]. On the other hand, the quantity of apo E carried in the particle may also be significant, provided that pre- β migrating VLDL from apo E₂ homozygotes, which are poorer in apo E than β -VLDL from the same subjects [39], are normally processed to LDL [8]. The studies with artificial substrates are far from conclusive since a suppression [40,41], an activation [42] or no effect [43] of apo E on LPL activity have been reported. Besides, a dual effect, i.e., inhibition or stimulation, has been found depending on whether apo E was free in the medium or complexed to the substrate emulsion [44]. Here we have observed that apo E-rich VLDL are less hydrolysed by LPL than apo E-poor VLDL, which suggests an inhibitory role for this apolipoprotein on LPL action, although the mechanism for this effect and its physiological significance remain to be clarified.

In summary, we have characterized three VLDL subfractions from normolipidemic human serum. All three bound to heparin-Sepharose at an ionic strength of 0.05 M NaCl/0.025 M MnCl₂, an ability probably conferred by their apo E. The subfraction richest in apo E and cholesterol and poorest in triacylglycerols showed the lowest degree of triacylglycerol hydrolysis by LPL and, hence, the lowest ability to deliver fatty acids to the adipose tissue. These differences do not seem to be due to changes in their apo C-II or triacylglycerol contents, but rather to their differences in apo E. Further studies are required to ascertain the precise mechanism that governs the efficiency of triacylglycerol hydrolysis by LPL in VLDL subfractions with different apo E contents.

Acknowledgements

This research was supported by grants from the Dirección General de Investigación Científica y Tecnológica (PMM 88/0049) and the Fondo de Investigaciones Sanitarias de la Seguridad Social (90/0269 and 92/0406), Spain. Dr G.T. Sáez was a recipient of a fellowship from the Fundación Juan March, Spain. We thank Carol F. Warren for editorial assistance.

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