Very–Low-Density Lipoprotein of Uremic Patients Is a Poor Substrate for Bovine Lipoprotein Lipase In Vitro

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Very–low-density lipoprotein (VLDL) from 10 hemodialysis patients and 10 healthy controls was studied with respect to the substrate characteristics for bovine milk lipoprotein lipase (LPL). Compared with the control subjects, the hemodialysis patients had significantly higher serum triglyceride and apolipoprotein B–associated apolipoprotein CIII concentrations (1.03 ± 0.31 *v* 1.98 ± 0.86 mmol/L and 0.004 ± 0.002 *v* 0.011 ± 0.005 g/L, respectively), lower serum high-density lipoprotein (HDL) cholesterol and apolipoprotein AI concentrations (1.33 ± 0.37 *v* 0.95 ± 0.31 mmol/L and 1.29 ± 0.25 *v* 1.09 ± 0.23 g/L, respectively), and lower postheparin plasma LPL activity (82 ± 24 *v* 35 ± 14 mU/mL). There were also significant increases in the relative fat content and diameter of VLDL particles from patients versus controls. VLDL was labeled with a fluorescent phospholipid analog, DHPE, and the rate of the lipolytic reaction with purified bovine milk LPL was estimated from the increase in fluorescence intensity at 490 nm. There was no significant difference between initial reaction velocities in the study groups, but VLDL particles from hemodialysis patients were lipolyzed to a significantly lesser extent than those from healthy controls (mean increase in fluorescence intensity after completion of the reaction, 95 ± 36 *v* 140 ± 43 arbitrary units). These results are in accordance with the accumulation of remnant particles reported to occur in uremia despite only a moderately increased serum triglyceride concentration.

THE DYSLIPOPROTEINEMIA of uremia is characterized by moderate hypertriglyceridemia,[1,2] low serum high-density lipoprotein (HDL) cholesterol,[3] and accumulation of remnant particles.[4-6] There is evidence that this is primarily caused by impaired catabolism of triglyceride-rich lipoproteins.[7] Hydrolysis of triglycerides by lipoprotein lipase (LPL) is the rate-limiting step in the catabolism of triglyceride-rich lipoproteins. Impaired triglyceride catabolism in uremic patients could be caused by decreased activity of LPL or by poor substrate function of the triglyceride-rich lipoproteins, very–low-density lipoprotein (VLDL) and chylomicrons. Accordingly, the decreased activity of postheparin plasma LPL in uremic patients reported by several groups[7,8] is probably an important etiologic factor for the hypertriglyceridemia in this patient category. However, there are many reports of compositional changes in the VLDL fraction of uremic patients, such as relative cholesterol enrichment,[9,10] increased apolipoprotein CIII concentration, and decreased apolipoprotein CII/CIII ratio.[11-13] Apolipoprotein CIII in uremic patients has also been found to be relatively rich in sialic acid,[13] a biochemical variant that has been associated with hypertriglyceridemia.[14] In addition, substances retained by the kidneys might interact with the surface of the lipoproteins, eg, by carbamylation. All these compositional changes might alter the substrate function of VLDL particles of uremic patients.

In the present study, rates of lipolysis of VLDL particles from hemodialysis patients and healthy controls were compared by the method described by Johnson et al,[15-17] using VLDL labeled with a fluorescent phospholipid analog as a substrate for bovine milk LPL.

SUBJECTS AND METHODS

Patients

Ten hemodialysis patients and 10 healthy controls were included in the study. There were five men and five women in each group. The median age of the patients was 55 years (range, 36 to 70), and controls, 45 years (range, 28 to 75). The patients had no other diseases known to affect triglyceride metabolism. They were on bicarbonate dialysis 4 to 6 hours three times per week. Dialysis-associated anticoagulation was achieved with unfractionated heparin. The patients were chosen to cover a spectrum of triglyceride concentrations representative of hemodialysis patients, ie, from normal to moderately elevated.

Blood Sampling and VLDL Isolation

Blood samples were collected after an overnight fast (12 hours), from hemodialysis patients 48 to 72 hours after dialysis. Heparin 50 IU/kg body weight was injected, and a blood sample was collected 15 minutes later for LPL activity analysis. The blood sample was immediately chilled on ice and centrifuged within 1 hour. Serum and plasma samples were stored at −20°C until analysis, with the exception of a 15- to 20-mL aliquot of preheparin-EDTA plasma, which was used for preparation of VLDL. This plasma was ultracentrifuged in Beckman Ti 50 rotor for 24 hours at 40,000 rpm at 8°C. VLDL was aspirated from the top of the tube and ultracentrifuged again under the same conditions at a density of 1.006 g/mL. The VLDL sample was then dialyzed for 24 hours against a 0.01-mol/L Tris chloride buffer (pH 7.4) containing 0.15 mol/L NaCl. The molecular weight cutoff of the dialysis membrane was 12 to 14 kd.

Lipolysis

VLDL was labeled with the fluorescent probe, *N*-(5-dimethylaminonapthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine ([DHPE] Molecular Probes, Eugene, OR). The molar ratio of VLDL triglyceride to DHPE was 40:1.[15,16] The mixture of VLDL and DHPE, the latter dissolved in chloroform, was vortexed for 15 seconds and sonicated in a water bath for 8

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Submitted February 25, 1994; accepted November 29, 1995.

Supported by the Swedish Medical Research Council (04966), the Medical Faculty, University of Lund, and Påhlssons Foundation.

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0026-0495/96/4506-0004$03.00/0

minutes at 23°C. The mixture was then allowed to equilibrate at 4°C for at least 1 hour before use. The test tubes were not covered, to allow the chloroform to evaporate.

The substrate mixture was composed of 0.3 μmol labeled VLDL triglycerides, 100 μL 20% fatty acid–free human serum albumin, and a buffer (0.15 mol/L NaCl and 0.05 mol/L Tris hydrochloride, pH 7.4) in a final volume of 1 mL. The cuvette was placed in a Perkin Elmer LS fluorometer at 37°C, and the fluorescence intensity was recorded (excitation 340 nm, emission 490 nm). The reaction was started by adding 30 μL purified bovine milk LPL. The increase in fluorescence intensity was monitored for 60 minutes. The initial velocity of the reaction was calculated from the ascending linear portion of the fluorescence intensity curve. The extent of lipolysis was expressed as the increase in fluorescence intensity after 60 minutes and the ratio of final to initial fluorescence intensities (F_{60}/F_0).

Analyses were performed in duplicate within 36 hours of the end of the VLDL isolation procedure. Plasma samples from a patient and a healthy control were ultracentrifuged in the same run. The corresponding VLDL samples were dialyzed in the same bath and labeled simultaneously, and the reactions were performed successively. Repeated analyses of the same VLDL sample stored at 4°C showed a durability of approximately 10 days. The interassay variation was less than 5%.

LPL Isolation

Purified bovine milk LPL was isolated and provided by Bengtsson and Olivecrona.[18] The specific activity was 500 μmol fatty acid/min · mg with triolein as substrate. LPL (0.26 mg/mL) was diluted 1:9 in 1.0 mol/L NaCl, 0.01 mol/L Tris hydrochloride, and 1 mg/mL bovine serum albumin and stored at −70°C.

Lipid, Lipoprotein, Apolipoprotein, and Lipase Analyses

Lipids were analyzed with enzymatic methods (Boehringer, Mannheim, Germany). Serum HDL cholesterol concentrations were measured after precipitation of VLDL cholesterol and low-density lipoprotein (LDL) cholesterol with dextran sulfate and magnesium chloride.[19] The analytical imprecision was less than 3%. Serum LDL cholesterol concentrations were calculated according to the Friedewald formula.[20]

Serum apolipoprotein AI and apolipoprotein B concentrations were measured by immunoturbidimetry with reagents from Roche (Basel, Switzerland).

Serum concentrations of apolipoprotein CIII, total and non–apolipoprotein B-associated, were analyzed by electroimmunodiffusion. Serum apolipoprotein B–associated apolipoprotein CIII concentrations were obtained by subtraction; serum apolipoprotein B–associated apolipoprotein E concentrations were obtained by the same procedure. Apolipoprotein CIII and E analyses were performed at Institut Pasteur (Lille, France).

VLDL total cholesterol, free cholesterol, triglyceride, and phospholipid concentrations were analyzed by enzymatic methods (Boehringer, and Wako, Neuss, Germany [phospholipids]). Esterified VLDL cholesterol concentrations were obtained by subtracting free cholesterol from total cholesterol. VLDL protein concentrations were analyzed using the Lowry method.[21] The relative VLDL content of all components (weight/weight) was calculated.

LPL activity was measured with a direct and selective method using sonicated [^{3}H]-trioleoylglycerol emulsion, stabilized by phosphatidyl choline, as substrate.[22] Albumin was added to the substrate after sonication. The reaction was performed at pH 8.0 with 0.15 mol/L NaCl and in the presence of serum. One milliunit of lipase activity represents the release of 1 nmol fatty acid per minute. The intraassay variation was less than 4%.

Electron Microscopy

Fresh VLDL particles from eight patients in each group were examined. They were negatively stained by sodium phosphotungstate and examined with a JEM 100 CX electron microscope (JEOL, Tokyo, Japan). Photomicrographs were taken at a magnification of 33,000. These were, in turn, photographically magnified by a factor of 7.4. The diameters of 100 free-standing particles were measured by a scale. To adjust for the flattening of the most triglyceride-rich particles during staining, the correction formula of Glomset et al[23] was applied to particles greater than 35 nm in diameter.

Statistical Methods

The data were evaluated with the Mann-Whitney *U* test and the Spearman rank correlation test.

RESULTS

Under our experimental conditions, fluorescence intensity increased rapidly after addition of bovine LPL. The reaction approached steady state after about 30 minutes (Fig 1). Values recorded after 60 minutes were only marginally higher than those at 30 minutes. Increases in fluorescence intensity were significantly lower in patients than in controls (Table 1). However, there was no significant difference between initial reaction velocities of the study groups. Initial fluorescence intensities were similar in both groups.

Patients had significantly higher serum triglyceride and apolipoprotein B–associated apolipoprotein CIII concentrations, lower serum HDL cholesterol and apolipoprotein AI concentrations, and lower plasma LPL activities than controls (Table 2). Serum triglyceride concentrations were directly correlated with apolipoprotein B–associated apolipoprotein CIII levels ($r = .64, P < .01$) and inversely correlated with plasma LPL activities ($r = -.51, P < .05$). There were no differences between serum total cholesterol, apoli-

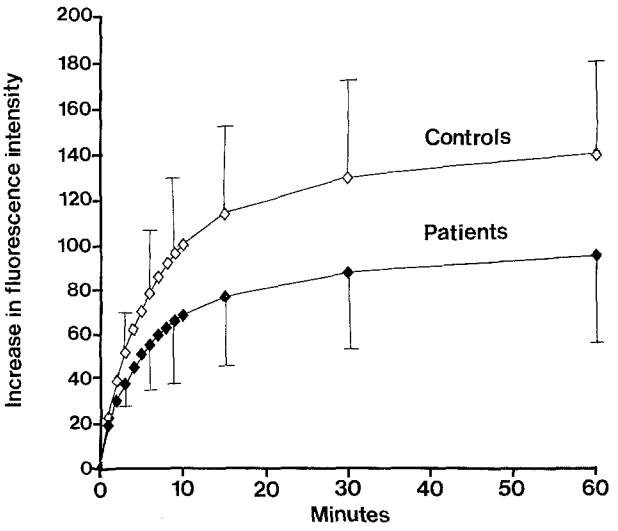

Fig 1. Increases in fluorescence intensity (arbitrary units) in DHPE-labeled VLDL from hemodialysis patients and healthy controls during incubation with bovine milk LPL in the presence of albumin (mean ± SD).

Table 1. Lipolysis of DHPE-Labeled VLDL From Hemodialysis Patients and Healthy Controls Induced by Bovine Milk LPL (mean ± SD)

Parameter	Patients	Controls	P
Initial velocity (U/min)	19.1 ± 4.5	22.4 ± 7.3	NS
Increase in F intensity after 60 minutes (U)	95 ± 36	140 ± 43	<.05
F_{60}/F_0	1.9 ± 0.3	2.2 ± 0.3	<.05

Abbreviations: U, arbitrary fluorescence unit; F, fluorescence at a given time (minutes).

poprotein B, and apolipoprotein B–associated apolipoprotein E concentrations of the two groups. The relative contents of free cholesterol, esterified cholesterol, and phospholipids were significantly higher and relative protein contents were slightly lower in VLDL from the patients. There was not a significant difference between the groups regarding relative VLDL triglyceride contents (Table 3).

VLDL diameters were significantly increased in patients as compared with controls, with a mean increase of 15% (Table 4). The total range of diameters was 15 to 68 nm. Patients had significantly more particles in the 51- to 68-nm range and significantly fewer particles in the 15- to 32-nm range (Table 4).

Correlations between increases in fluorescence intensity and other variables in all study subjects are shown in Table 5. When tested separately for each group, most of these relations remained significant in the control group (Table 5), but in the patient group, correlations between increases in fluorescence intensity and other variables did not reach statistical significance. There was a significant inverse correlation between initial reaction velocities and serum triglyceride concentrations in all study subjects ($r = -.50$, $P < .029$) and in healthy controls ($r = -.77, P < .022$), but not in uremic patients ($r = -.31$, NS).

DISCUSSION

Lipolysis of VLDL particles from hemodialysis patients and healthy controls was monitored in vitro. This was achieved using a fluorescent phospholipid analog (DHPE), which can be incorporated into VLDL and hydrolyzed by LPL. A marked increase in fluorescence intensity occurs when lyso-DHPE is liberated from DHPE-VLDL and bound by albumin.[16] The increase in fluorescence intensity has been shown to correlate well with the release of fatty acids,[15] thus providing a sensitive method for monitoring the lipolysis of triglyceride-rich lipoproteins. There was no significant difference between the study groups in terms of initial reaction velocities. On the other hand, the extent of lipolysis of VLDL particles was significantly less for hemodialysis patients versus controls.

Table 2. Serum Lipid, Lipoprotein, and Apolipoprotein Concentrations and LPL Activities of Hemodialysis Patients and Healthy Controls (mean ± SD)

Variable	Patients	Controls	P
Cholesterol (mmol/L)	5.1 ± 1.1	5.5 ± 1.0	NS
HDL cholesterol (mmol/L)	0.95 ± 0.31	1.33 ± 0.37	<.05
LDL cholesterol (mmol/L)	3.2 ± 1.0	3.7 ± 1.0	NS
Triglycerides (mmol/L)	1.98 ± 0.86	1.03 ± 0.31	<.05
Apo AI (g/L)	1.09 ± 0.23	1.29 ± 0.25	<.05
Apo B (g/L)	1.04 ± 0.35	1.00 ± 0.26	NS
Apo B–associated apo CIII (g/L)	0.011 ± 0.005	0.004 ± 0.002	<.01
Apo B–associated apo E (g/L)	0.011 ± 0.006	0.013 ± 0.010	NS
LPL activity (mU/mL)	35 ± 14	82 ± 24	<.01

Abbreviation: apo, apolipoprotein.

Table 3. Composition of VLDL From Hemodialysis Patients and Healthy Controls (% wt/wt, mean ± SD)

Variable	Patients	Controls	P
Triglyceride	53.1 ± 2.3	47.3 ± 12.0	NS
Free cholesterol	6.3 ± 0.7	5.0 ± 0.6	<.05
Esterified cholesterol	6.5 ± 1.8	4.7 ± 1.4	<.05
Phospholipid	15.2 ± 1.2	13.1 ± 1.7	<.05
Protein	18.9 ± 5.0	29.9 ± 13.9	NS

VLDL particles from hemodialysis patients contained relatively more cholesterol and phospholipids than those from healthy controls. They were also increased in diameter. The relative contents of free cholesterol and phospholipids, as well as particle diameters, correlated inversely with the extent of lipolysis. Thus, an increase in cholesterol and phospholipids at the VLDL surface and an increase in particle size might interfere with the lipolytic process. The same applies to an increase in serum apolipoprotein B–associated apolipoprotein CIII concentration. However, causal connections cannot be claimed. The fact that correlations between increases in fluorescence intensity and other variables were not statistically significant in the patient group probably depends on the multifactorial nature of uremic dyslipoproteinemia, in combination with the relatively small size of the group.

Since the incubations were performed with an excess of enzyme, the effective substrate concentrations (ie, VLDL surface areas) were rate-limiting. The mean difference in diameter of 15% equals approximately a 50% difference in VLDL particle volume and a 30% difference in VLDL particle surface area. There would therefore be a difference in total VLDL surface areas available for interaction with the enzyme between reaction mixtures containing the same amount of VLDL triglycerides from hemodialysis patients and controls. The mean total VLDL surface area was estimated to be 15% smaller in incubations with patients' samples, allowing LPL less access to VLDL lipids. This can account for the tendency for slower initial reaction velocity in the patients as compared with the controls, a difference amounting to 17%, although not statistically significant. After taking this into consideration, initial reaction velocities seemed to be almost identical in the study groups. The reactions were allowed to proceed to completion. The total

Table 4. Mean Size and Size Distribution of VLDL From Hemodialysis Patients and Healthy Controls (mean ± SD)

Variable	Patients	Controls	P
Mean size (nm)	37.3 ± 3.3	32.5 ± 3.7	<.05
15-32 nm (%)	24 ± 21	46 ± 23	<.05
33-50 nm (%)	67 ± 20	52 ± 22	NS
51-68 nm (%)	9 ± 5	2 ± 1	<.05

Table 5. Correlations Between VLDL Lipolysis In Vitro and Lipid, Lipoprotein, and Apolipoprotein Concentrations, LPL Activities, and Relative Contents of VLDL in Hemodialysis Patients and Healthy Controls

Variable	All Subjects		Controls	
	r*	P	r*	P
Serum cholesterol	−.34	NS	−.49	NS
Serum HDL cholesterol	.38	<.05	.27	NS
Serum LDL cholesterol	−.36	NS	−.67	<.05
Serum triglycerides	−.40	<.05	−.64	<.05
Serum apo B–associated apo CIII	−.49	<.05	−.68	<.05
Serum apo B–associated apo E	.12	NS	−.18	NS
LPL activity	.03	NS	−.70	<.05
VLDL triglycerides	−.10	NS	−.13	NS
VLDL free cholesterol	−.65	<.01	−.69	<.05
VLDL esterified cholesterol	−.32	NS	−.03	NS
VLDL phospholipid	−.54	<.05	−.58	<.05
VLDL protein	.38	NS	.25	NS
VLDL diameter	−.73	<.01	−.57	NS

NOTE. VLDL lipolysis represents the increase in fluorescence intensity after 60 minutes incubation of DHPE-labeled VLDL with bovine milk LPL.

Abbreviation: apo, apolipoprotein.

*Spearman rank correlation coefficient.

VLDL surface area therefore would not be expected to influence the extent of lipolysis. Thus, the difference in the extent of lipolysis seems to be due to the inherent characteristics of VLDL particles.

Postheparin plasma LPL activities were significantly lower in hemodialysis patients, which probably resulted in a retardation of the whole lipolytic process in vivo. VLDL isolated from the plasma of study objects had been exposed to lipolytic activity in vivo; in fact, most of the VLDL particles must have been in a phase somewhere between secretion and final lipolysis. Due to lower plasma LPL activities in vivo, it is reasonable to assume that VLDL isolated from the patients contained a greater proportion of particles in a state of less advanced lipolysis. A study of nascent VLDL particles would probably have shown a still greater difference regarding the extent of lipolysis. It is more difficult to predict the effect on initial reaction velocities.

Thus, the results of the in vitro experiments cannot be directly extrapolated to the in vivo situation. After considering the possible sources of error, it can be concluded that VLDL particles from the hemodialysis patients were inferior substrates for bovine LPL regarding the extent of lipolysis. Initial reaction velocities of the study groups are more difficult to compare, but at any rate, the difference did not seem to be as pronounced as that for the degree of lipolysis.

Dyslipoproteinemia in uremia is characterized by the unique combination of accumulation of remnant particles[4-6] and only moderate hypertriglyceridemia. The lipolytic pattern of VLDL particles from uremic patients found in the present in vitro study, ie, resistance to final lipolysis despite unchanged initial lipolysis, fits well with the features of uremic dyslipoproteinemia.

ACKNOWLEDGMENT

We are indebted to Tomas Olivecrona for providing purified bovine milk LPL, and Erik Carlemalm for assistance with electron microscopy. Anette Jutzeler and Pascal Lebel gave excellent technical assistance.

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