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Hydrolysis of Human Very Low Density Lipoprotein Triglycerides in Rat Heart

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Abstract The kinetic constants for human very low density lipoproteins (VLDL) of membrane-supported lipoprotein lipase (LPL) of the rat were studied. The VLDL substrate (Sf 20-400) was obtained from the plasma of healthy human subjects with normolipidemia and with normal patterns of VLDL apoprotein peptides in isoelectric focusing electrophoretogram.

Triglycerides of human VLDL were hydrolyzed by LPL on the vascular surface of the isolated rat heart. The calculated maximal reaction velocity (Vmax) and the apparent Michaelis constant (Km) were 0.037 ± 0.006 μmol $min^{-1}ml^{-1}$ and 0.060 ± 0.004 mM (Mean$\pm$S.D.), respectively. These values were very close to the corresponding values obtained using rat VLDL as a substrate. This system provides a means to study possible kinetic abnormalities of the triglyceride-rich lipoproteins on the vascular surface of the heart of hyperlipidemic patients.

Key Words: Lipoproteins; lipoprotein. Lipase; hyperlipidemia, triglyceride, kinetics. Heart; hyperlipidemia

Introduction

Determination of the LPL activity is important in the study of the abnormalities of lipid metabolism in hyperlipidemic patients. The LPL activity has so far been determined only with the solubilized form of the enzyme that was released after the injection of heparin from the vascular surface into the blood stream. The kinetic properties of the membrane-supported LPL may differ from those of the solubilized LPL for several reasons: 1) the steric configulation of the enzyme may be altered, affecting the properties of the active site, 2) the enzyme-substrate reaction on the membrane surface may be diffusion limited, 3) the electrical properties of the membrane may affect the reaction rate.

Recent experiments with the tissue perfusion system have shown that the LPL on the vascular surface of the rat heart and in the rat adipose tissue follows Michaelis-Menten kinetics when triglyceride-rich lipoproteins are used as a substrate. Therefore, the perfusion system provides a means to study properties of membrane-bound LPL under physiological conditions[1].

Meanwhile, the hydrolysis of lipoprotein-triglycerides is accompanied by the transfer of a cofactor protein, apoprotein C-II (apo C-II), from high density lipoproteins (HDL) to triglyceride-rich lipoproteins[2-3]. The catabolism of triglyceride-rich lipoproteins is greatly impaired in the patients who lack apo C-II. The LPL from several sources including rat and human post-heparin plasma, rat adipose tissue and cow's milk is activated by apo C-II[4]. These results indicate that human triglyceride-rich lipoproteins containing the normal amount of apo C-II serve as an active substrate for rat heart LPL. We therefore studied the kinetic properties of human VLDL in the perfusion system and compared the values from healthy subjects and hyperlipidemic patients.

In this experiment, kinetic constants, Vmax and apparent Km for VLDL from healthy human were determined using LPL on the vascular surface of isolated and perfused rat hearts. The VLDL apoprotein peptides were also analyzed. The results on hyperlipidemic patients will be reported in a separate paper.

Materials and Methods

Preparation of Lipoprotein Substrate

Lipoprotein donors were five healthy human subjects, 30 to 55 years old, who had undergone medical examinations for the preceding 2-8 years and were diagnosed as healthy. Their serum cholesterol and triglyceride levels had been measured twice a year for the preceding five years. Their diet had also been evaluated once every year. Blood samples were drawn from the subjects after an overnight fast. After an initial ultracentrifugation of plasma at 0.61×10^6 g-min[5] to remove large particles ($Sf>400$), VLDL were isolated by a second centrifugation at 1.2×10^8 g-min[6]. To avoid the contamination by other lipoprotein class, centrifugation was repeated twice under the same conditions. The VLDL-triglycerides were labeled by an exogenous isotopic labeling procedure:[7] 10μCi glyceryl-tri-[9, 10(n)-^{3}H] oleate (100-500 mCi/mmol Radiochemical Centre, Amersham) was dissolved in 1 ml of dimethylsulfoxide (Sigma, St. Louis, Mo). The solution was added with constant stirring to 4 vols of 0.15 M NaCl containing 0.1% (w/v) sodium EDTA (pH 7.4). The solution was then mixed with the same volume of VLDL solution and incubated for 3 h at 37°C. The labeled VLDL were dialyzed overnight against saline EDTA and then reisolated by ultracentrifugation as described above. Finally, the VLDL were dialyzed against 500 ml of Krebs-Henseleit bicarbonate buffer. The composition of the VLDL and the specific activities of the VLDL-triglycerides were determined.

Analysis of VLDL Apoprotein Peptides

The VLDL apoprotein peptides, including cofactor protein for VLDL, were analyzed by isoelectric focusing electrophoresis[8-10]. The VLDL were isolated from the sera of the subjects by preparative ultracentrifugation under the conditions described above. This was repeated once more to eliminate contamination with other plasma proteins. The VLDL solutions, in which protein was concentrated to at least 3 mg/ml, were delipidated by cold ethanol-ether (3:1) and ether[11]. The extracted VLDL apoproteins were dissolved in 1% sodium decyl sulfate in 0.11 M Tris and 20% sucrose, pH 8.3, containing 0.1 vol of β-mercaptoethanol to give a concentration of 12-15 mg/ml. A hundred μg of VLDL was applied to the 7% polyacrylamide gels (8×0.7 cm) containing 8 M urea and 1.6% Ampholine (pH 3.5-7.0, LKB, Bromma, Sweden), after focusing at 100 V for 30 min. Focusing of 12 gels was done at 10°C for 1 h at 200 V and then for 3 h at 400 V. The gels were stained with Comassie Brilliant Blue G (Sigma, St. Louis, Mo) [12] and the optical density of each component was measured at 560 nm by spectrophotometric scanning. Percentage contribution for each of the densitometric areas of the VLDL peptides was quantified[10].

Perfusion Procedure

The procedure described by Fielding[1] was used with a slight modification. Hearts from 300-350 g male Sprague-Dawley rats were perfused with a modified recirculating Langendorff aparatus. All glassware and tubing were siliconized. A 12 ml perfusion medium contained 3.0 ml of dialyzed 15% (w/v) bovine serum albumin (Fract. V, Sigma), 0.5 mlof three-fold concentrated VLDL free-plasma, 0.4 ml of 0.1 M glucose, Krebs-Henseleit bicarbonate buffer gassed with 95% O_2 and 5% CO_2 and

requisite volume of VLDL substrate. The medium (pH 7.4) was passed at a flow rate of 5-7 ml/min through the heart placed in a perfusion chamber which was maintained at 37°C. In each experiment, the heart vigorously contracted with regular rhythm, ranging between 120-160/min during the perfusion period. The total perfusion time was about 40 min. Duplicate 0.2 ml samples of perfusion medium were drawn every 2 min for about 14 min. The samples were mixed with Dole's mixture and then VLDL-triglycerides were extracted with heptane. ^{3}H in the fraction of neutral lipids and free fatty acids were measured by liquid scintillation spectrometric scanning. The elimination curve for ^{3}H-neutral lipids were mainly determined by least squares analysis from double reciprocal plots. The rate of triglyceride hydrolysis was calculated as μmol triglycerides $min^{-1}ml^{-1}$ of perfusate. The kinetic constants were determined by the equations;[1)]

$$S_o - S_t = k_c Et + Km \ln(S_t/S_o)$$

where S_o and S_t are substrate concentrations at 0 and t min, and k_cE is Vmax. For low substrate concentration, $Km \gg S_o$

$$1/t \ln(S_o/S_t) = k_cE/v_t Km$$

For high substrate concentration, $S_o \gg Km$,

$$(S_o - S_t)/t = k_cE/v_t$$

v_t is total volume of enzyme system.

Results

Diets, Serum Lipids and Lipoprotein Values

The average intake of nutrients by the five healthy persons during the preceding five years is shown in Table 1. Their mean serum total cholesterol and triglyceride levels and composition of the VLDL are shown in Table 2.

Content of Apoprotein Peptides in VLDL

Four major protein components of apo C and three components of apo E were evident by isoelectric focusing electrophoresis. An electrophoretogram of VLDL apoprotein peptides is shown Fig. 1. In several cases, two additional faint bands were visible. One identified as apo C-II_2[9)], was situated between C-III_1 and C-III_2, and the other was anionic to C-III_2. Apo E patterns were normal. Apo E_4 was not observed in any of the subjects. There were no subjects with abnormal apoprotein peptides.

Table 1 Diet of the Five Subjects (Component of Diet; g/day)

Carbohydrate	Protein	Fat	
		vegitable	animal
365 ±30	78 ±9	13 ±4	25 ±5

Mean±S.D.
The diet of the subjects has been evaluated once every year for the past 5 years. Mean daily caloric intake was 2395±102 Cal. (n=25 in aggregate).

Table 2 Serum Lipid Level and Composition of VLDL

Serum (mg/dl)		VLDL: Sf 20-400 (%)			
Ch.	TG	Ch.	TG	PL	Prot.
219 ±22	131 ±45	16.2 ±0.9	53.6 ±3.5	19.5 ±1.3	10.9 ±2.2

Mean±S.D.
Ch: total cholesterol, TG: triglyceride, PL: phospholipid, Prot.: protein. (n=5).

Table 3 Percentagge Distribution of Lpo C and Apo E Components of VLDL in Human Subjects

$C\text{-}III_0$	$C\text{-}II_1$	$C\text{-}III_1$	$C\text{-}III_2$	E_1	E_2	E_3
11.6 ±1.0	17.9 ±1.7	23.5 ±1.5	20.7 ±1.1	7.8 ±0.6	6.1 ±0.3	12.5 ±1.0

Mean±S.D. (n=5).

Kinetic Constants of Human VLDL for LPL of Rat Heart

Hydrolysis of VLDL-triglycerides was zero-order at the initial substrate concentration grater than 1.0μmol/ml and first-order at the concentration less than 0.01μmol/ml. The elimination curves are shown in Fig. 2. During the perfusion, triglyceride hydrolysis was evident because ^{3}H in free fatty acid increased, although free fatty acid was partly

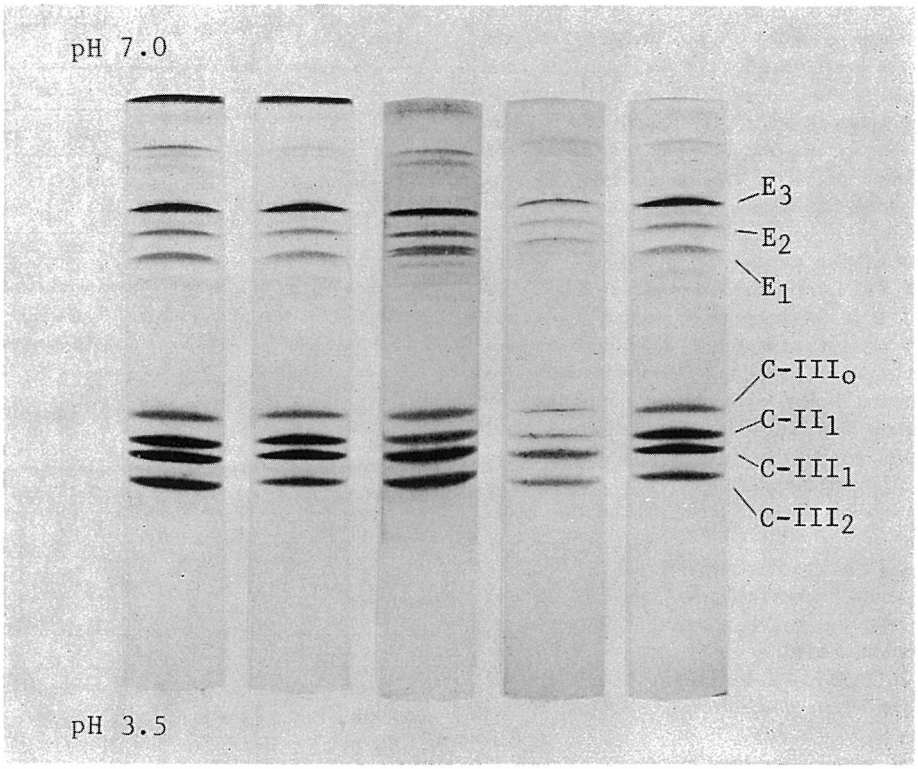

Fig. 1 Analytical isoelectric focusing electrophoretogram of VLDL apo C and apo E in 5 healthy human subjects. Isoelectric points of apo C components are between 4.91 and 4.50, and apo E components are between 5.60 and 6.05 (pH), respectively.

taken up into the heart. The mean Vmax, measured in five subjects, was 0.037±0.006 μmol $min^{-1}ml^{-1}$ and the mean apparent Km measured in three subjects was 0.060±0.004 mM.

Discussion

LPL is activated in the presence of apo C-II. It has been indicated by in vitro experiments that the lipoprotein-triglycerides are hydrolyzed after the transfer of apo C-II from HDL to substrate[2-3]. However, apo C-II is not a rate-limiting factor for hydrolysis of lipoprotein-triglycerides in vitro until about 75% of the total activator has been lost from the original substrate[13]. The maximal rate of triglyceride hydrolysis is achieved with much smaller quantities of apo C-II than those that are bound by lipoprotein particles[14]. In addition, the lipid composition of the substrate and the relative proportions of intact and remnant lipoproteins may regulate the rate of hydrolysis of lipoprotein-triglecerides, as observed when one-third of substrate triglecerides has been hydrolyzed[13]. In the present study, VLDL was prepared from healthy humans. The lipid composition and the pattern of VLDL apoprotein peptides, including apo C-II on isoelectric focusing electrophoretogram, were normal, although the content of apo C-II was unknown. Furthermore, the contribution of the remnant lipoproteins to the rate of triglyceride hydrolysis should be little, beca-

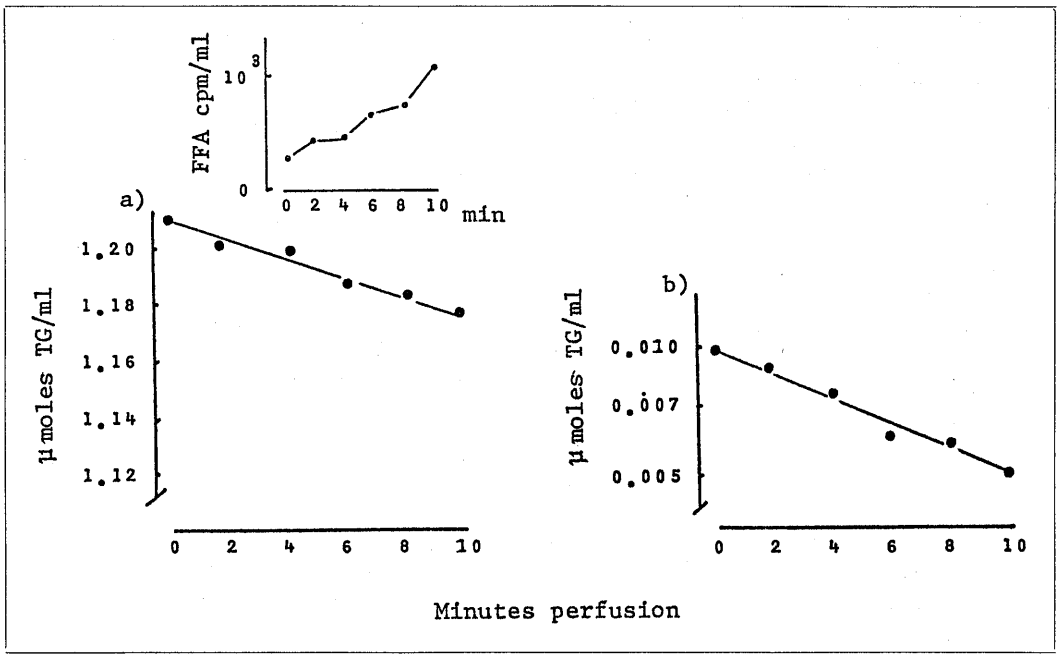

Fig. 2 Removal of VLDL-triglycerides by the rat heart. Sample was obtained from healthy human. Flow rate of the substrate was 6 ml/min. a): zero-order removal rate at high substrate concentration. b): first-order removal rate at low substrate concentration.

Insert: increase of FFA during the perfusion. Increment of ^{3}H-FFA was about one-third of decrement of ^{3}H-triglyceride.

use the amount of the hydrolyzed triglycerides is much smaller than one-third of the initial concentration of triglycerides in this experiment. Therefore, it appears that there are no contributions of above mentioned inhibitory factors to LPL activity.

In our perfusion system, human VLDL proved to be an appropriate substrate for rat heart LPL. The calculated kinetic constants, Vmax and Km, are $0.037 \pm 0.006 \mu$mol $min^{-1}ml^{-1}$ and 0.060 ± 0.004mM, respectively The kinetic interpretation of activities of the enzyme suppoted in the solid matrix has been presented[1,15]. Substrate partition affects the apparent Km. The Km is inversely proportional to the substrate difusion constant. In diffusion limited reaction, apparent Km will decrease as the flow rate in the reaction column increase. However, in an experiment with perfusion of isolated rat hearts, Km is independent of flow rate in the physiological range (4-10 ml/min) at a high affinity enzyme site like heart LPL[16]. The partition effect is also independent of flow rate. Furthermore, the Km for small particles of rat VLDL (Sf 40-100) did not significantly differ from that for larger particles of rat VLDL (Sf 100-400)[13].

Therefore, it would be reasonable to compare the Km value of human VLDL in our experiment with that of rat VLDL (Km 0.06 mM), determined under the same experimental condition. Although the sizes of VLDL particles ranged between Sf 20-400 in our study, Km values were very close between human and rat VLDL.

On the other hand, Vmax is not partition- or diffusion-limited. Substrate particle size

contributes to catalytic rate. As shown previously, the Vmax of rat chylomicrons is 0.07 μmol $min^{-1}ml^{-1}$ for LPL supported on the vascular surface of rat heart[16]. Since the actual amount of the lipase active on vascular surface is unknown in our perfusion system, catalytic rates can not be directly determined. Catalytic rates for high affinity LPL solubilized in solution, have been shown as the following[13]; Chylomicrons: 3.16×10^3 min^{-1}, large VLDL (Sf 100-400) : 1.8×10^3 min^{-1}, and small VLDL (Sf 40-100) : 1.1×10^3 min^{-1}. Assuming that the amounts of the enzyme are the same, Vmax for these lipoproteins should be proportional to their catalytic rates. The Vmax for healthy human VLDL (Sf 20-400) was 0.037 μmol $min^{-1}ml^{-1}$. This value is roughly half that of rat chylomicrons. Assuming that isolated rat hearts have a constant amount of LPL, the catalytic rates of human and rat VLDL are similar to each other.

Hydrolysis among the triglyceride-rich lipoproteins is competitive. Hydrolysis rate of human VLDL (Sf 20-400) may partly vary with changing lipid and apoprotein composition of the lipoprotein particles. But, the results of our study indicate that the kinetic properties for human VLDL at least of high affinity LPL, are similar to those for rat VLDL. The perfusion system described in this paper should facilitate the study of kinetic abnormalities of human triglyceride-rich lipoproteins, especially of membrane-bound LPL, in hyperlipidemic subjects.

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