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Inactive but Dimeric Form of Lipoprotein Lipase in Human Plasma

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Active lipoprotein lipase (LPL) is known as a noncovalent homodimer of identical subunits, and dissociation of the dimer to a monomeric form renders the lipase inactive. In this study, the oligomerization status of LPL in human and rat plasma was investigated. The LPL activity was barely detectable in the control rat and human plasma. After the injection of heparin, the total lipolytic activity of plasma was rapidly increased, and reached its maximum in 30 min. Changes of the LPL protein correlated well with those of lipolytic activity. The LPL protein that is released by heparin into both human and rat plasma was active and dimeric in the sucrose density gradient ultracentrifugation. In control rat plasma, LPL was inactive, and a great fraction was present as an aggregate. However, the inactive LPL protein in the control human plasma retained the dimeric state, indicating that dimerization can be an entity independent of the catalytic activity of LPL. The released LPL is transported as a complex with lipoproteins in plasma. Lipoprotein profiles, determined by NaBr ultracentrifugation, exhibited typical LDL- and HDL-mammal patterns in humans and rats, respectively, with a smaller amount of the LDL fraction observed in rats. The difference in the lipoprotein profiles might influence the fate of the released LPL in plasma.

Keywords: Lipoprotein lipase, Dimerization, Heparin

Introduction

Lipoprotein lipase (LPL) is an acylglycerol hydrolase (EC 3.1.1.34) that hydrolyzes the triglyceride component of circulating chylomicron and very low density lipoprotein (VLDL) into two fatty acids and monoacylglycerol (Bensadoun, 1991). The enzyme is found in extrahepatic tissues including adipose tissues, cardiac and skeletal muscles, lactating mammary gland, lung, spleen, and brain (Braun and Severson, 1992). In these tissues the enzyme catalyzes the rate-limiting step for lipid acquisition from circulating triglycerides. The deficiency, or dysfunction of LPL, has been found in association with the pathogenesis of hypertriglyceridemia (Eckel, 1989).

LPL is produced by parenchymal cells in extrahepatic tissues, and then transferred to capillaries, where it acts (Braun and Severson, 1992). LPL is attached to the luminal surface of vascular endothelial cells via an interaction with membrane-bound heparan sulfate. It is released into the blood stream by heparin (Saxena *et al.*, 1991). In normal plasma, the LPL activity is not detectable (Bensadoun, 1991). Some studies in animals indicate that there is a continuous dissociation of the enzyme from the endothelium to the blood (Bagby, 1983). Generally, the released LPL was transported as an attached form to lipoproteins in plasma and taken up by the liver (Vilaro *et al.*, 1988; Karpe *et al.*, 1998).

Active LPL is known as a noncovalent homodimer of identical subunits (Garfinkel *et al.*, 1983). Dissociation of the dimer to the monomeric form renders the lipase inactive (Osborne *et al.*, 1985). The active, dimeric form of lipase also binds to heparin with a higher affinity than the inactive, monomeric form does (Liu *et al.*, 1993). Dimerization of LPL occurs early in the endoplasmic reticulum (Park *et al.*, 1997). Most of the LPL protein in adipocytes is present in the dimeric state (Park *et al.*, 1995). Until now, the dimeric form of LPL was considered as an active one that showed a high affinity for heparin.

In this study, we investigated the oligomerization status of LPL in human and rat plasma. We also observed the presence of the inactive, but dimeric form of LPL in human plasma.

Materials and Methods

Post-heparin plasma Post-heparin human plasmas from normal healthy adults, and patients with open heart surgery, were obtained 15 min after the injection of 3 mg/kg body weight of heparin (Vilella *et al.*, 1993). Rat plasma was also obtained under the same conditions. Blood was drawn in EDTA-containing tubes, and kept at 4°C until the separation of plasma.

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Assay of plasma lipolytic activity The lipolytic activity of plasma was measured, as described by Park *et al.* (1995). A stock triacylglycerol emulsion that contained 5 mCi of tri[9,10(n)-^{3}H]oleoylglycerol (Amersham), 1.13 mmol of trioleoylglycerol, 60 mg of 1-α-phosphatidylcholine (bovine liver), and 9 ml of glycerol was prepared according to the method of Nilsson-Ehle & Schotz (Nilsson-Ehle and Schotz, 1976). Before assay, 1 volume of the stock emulsion, 19 volume of 3% BSA in 0.2 M Tris-HCl buffer (pH 8.1), and 5 volume of heat-inactivated fasted rat serum (heated at 60°C for 30 min), were mixed and incubated for 15-30 min. For assay, 100 μl of this activated substrate mixture was added to the same amount of the enzyme solution. It was then incubated at 37°C for 60 min. Released fatty acids were extracted, and its radioactivity was measured. One mU of lipolytic activity represents the release of 1 nmol of fatty acid per min.

Immunoblotting of LPL LPL, from an aliquot of plasma, was concentrated using heparin-Sepharose (Pharmacia LKB) (Park *et al.*, 1996). After the addition of enough heparin-Sepharose that was equilibrated with 20 mM Tris, 0.3 M NaCl, and 1 M glycerol (pH 7.4), the mixture was kept on ice for 30 min with occasional mixing. It was then washed 5 times with an equilibration buffer, mixed with an electrophoresis sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.8), and boiled for 5 min.

Proteins were separated by SDS-PAGE with 10% resolving and 3% stacking acrylamide gels (Laemmli, 1970), then transferred to a nitrocellulose membrane. The membranes were blocked with 2% BSA. It was then reacted overnight with anti-bovine LPL chicken IgG (kindly supplied by Dr. Thomas Olivecrona in the Department of Physiological Chemistry, University of Umeä, Umeä, Sweden) and with alkaline phosphatase-conjugated anti-chicken IgG rabbit antiserum for 2 h. The color was developed in a BCIP/NBT solution (Bio-Rad).

Sucrose gradient ultracentrifugation The dimerization status of LPL was determined by sucrose density gradient ultracentrifugation, as described previously (Park, *et al.*, 1995). Adipose tissues were homogenized with the lysis solution (20 mM Tris, 100 mM NaCl, and 1% Triton X-100, pH 7.4) using a Polytron homogenizer. The supernatant was then centrifuged at 15,000 *g* for 20 min at 4°C (Park, *et al.*, 1996). An aliquot of tissue homogenate or plasma was mixed with sedimentation markers, bovine serum albumin (Mr = 65,400, S = 4.1), and yeast alcohol dehydrogenase (Mr = 150,000, S = 7.6). It was then layered on the top of a linear gradient of 5-20% (w/v) sucrose in a buffer that contained 20 mM 2-(*N*-morpholino)ethanesulfonic acid, 100 mM NaCl, 30 mM Tris-HCl, 1 mM $CaCl_2$, and 0.1% Triton X-100, pH 7.6 (3.6 ml) that was overlaid on 0.2 ml of 40% sucrose. The samples were centrifuged at 34,000 rpm for 16 h at 4°C. The fractions of 0.28 ml were collected from the bottom of the tube. Western blotting of the LPL protein was performed for each fraction.

NaBr gradient ultracentrifugation of plasma lipoproteins Plasma lipoproteins were separated by a NaBr density gradient ultracentrifugation in a swinging bucket rotor (Kelley and Kruski, 1986). The inner surface of the ultracentrifugation tube (5 ml) was made wettable with polyvinyl alcohol, described by Pitas & Mahley (Pitas and Mahley, 1992). The plasma adjusted to the density of 1.31 g/ml with NaBr (0.5 ml) was placed at the bottom of the tubes. Then the discontinuous gradient was formed above the plasma by layering the NaBr solutions with the density of 1.210, 1.063, 1.019 and 1.006 g/ml consecutively at the amount of 1.2, 1.5, 1.3 and 0.5 ml each. Each solution was pipetted down the inside of the tube (held at a 45 degree) with a slow continuous flow with great care to prevent the mixing.

Centrifugation was performed at 34,000 rpm for 16 h at 20°C in a Sorvall SW-650 swinging bucket rotor (DuPont-Sorvall, OTD-75B). The centrifuge tube was punctured from the bottom and a saturated NaBr solution was pumped into the bottom of the tube. About 25 fractions of 200 μl were collected from the top.

Triglyceride, cholesterol and protein determination Triglyceride (Wahlefeld, 1974) and cholesterol (Siedel *et al.*, 1983) were measured by enzymatic methods (Asan, Korea). Proteins were determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin as a reference standard.

Results

After the injection of heparin, the change of lipolytic activity in post-heparin human plasma was determined (Fig. 1). The total lipolytic activity of pre-heparin plasma was hardly detectable, but increased rapidly after the injection of heparin. It reached the maximum of 11.07 ± 2.94 mU/ml at 30 min. After that it began to decrease. It reached the baseline value in 4 h. Hepatic triglyceride lipase, in addition to LPL, was released into the plasma by heparin (Henderson *et al.*, 1993). To determine the increase of LPL among the total lipolytic

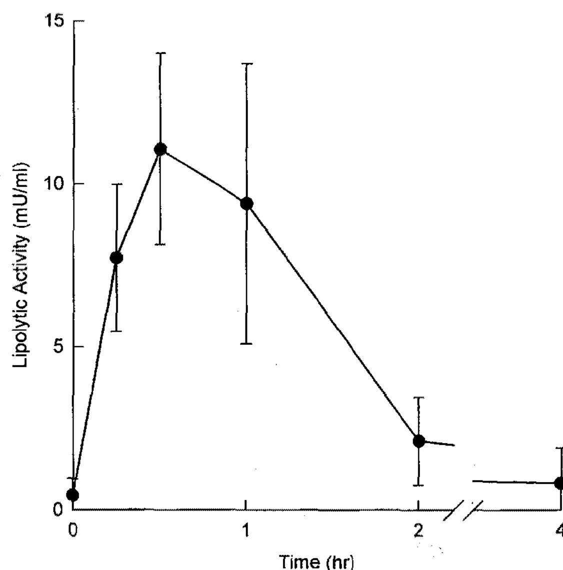

Fig. 1. Change of total lipolytic activity of human plasma after heparin injection. Post-heparin human plasma was obtained at various time intervals after the injection of 3 mg/kg body weight of heparin. Its lipolytic activity was determined, described under Materials and Methods.

Fig. 2. Change of LPL protein in human plasma after heparin injection. Post-heparin plasma was obtained, described in the Legend of Fig. 1, and the LPL protein was immunoblotted.

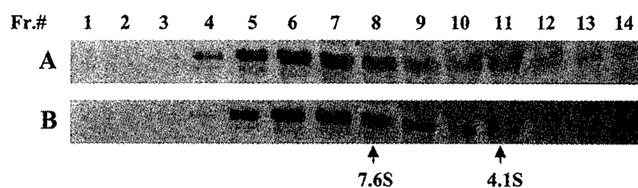

Fig. 3. Distribution of LPL proteins in sucrose density gradient ultracentrifugation fractions of pre- (A) and post-heparin human plasma (B). Aliquots of pre- and post-heparin plasma, obtained at 0 and 15 min after the injection of 3 mg/kg body weight of heparin, were resolved by 5-20% sucrose gradient ultracentrifugation as described in Materials and Methods. The LPL protein of each fraction was resolved by SDS-PAGE and immunoblotted. Arrows indicate the fractions that contain sedimentation markers, BSA (4.1 S) and yeast alcohol dehydrogenase (7.6 S), respectively.

activity, the LPL proteins were separated by SDS-PAGE and immunoblotted (Fig. 2). The change of LPL protein was similar to that of total lipolytic activity, reaching the maximum at 30 min. The LPL subunit showed a molecular weight of 57 kDa in SDS-PAGE. In spite of no detectable LPL activity, a significant amount of the LPL protein was observed in pre-heparin plasma.

The oligomerization status of human plasma LPL was determined using 5-20% sucrose density gradient ultracentrifugation. As shown in Fig. 3B, most of the LPL protein in post-heparin plasma was found in the 6th and 7th fractions from the bottom. The peak activity of yeast alcohol dehydrogenase, a sedimentation marker with a molecular weight of 150 kDa, was found at the 8th fraction from the bottom. The activity was found at the same fractions as LPL proteins. The same procedure was performed with pre-heparin human plasma (Fig. 3A). Most of the LPL protein in pre-heparin plasma was found at the same fractions as post-heparin plasma LPL. This indicates that the released LPL in normal plasma was not degraded, and still retained the dimeric state. However, no activity was detected from each fraction.

These results of human plasma were compared with those of rat plasma. In rats, most of the LPL in post-heparin plasma was observed at the dimeric fractions (Fig. 4B). The pattern of distribution was similar to that of human post-heparin plasma LPL. However, in pre-heparin rat plasma (Fig. 4A), some of the LPL was found in dimeric fractions, but a large fraction of the LPL protein was found at the bottom as an aggregate.

The free form of LPL, released from the endothelial cell wall to the plasma, was reported to bind with apo-B

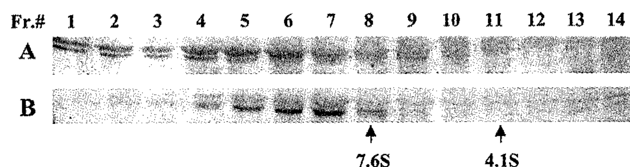

Fig. 4. Distribution of LPL proteins in sucrose density gradient ultracentrifugation fractions of pre- (A) and post-heparin rat plasma (B). Aliquots of pre- and post-heparin plasma, obtained at 0 and 15 min after the injection of 3 mg/kg body weight of heparin, were resolved by 5-20% sucrose gradient ultracentrifugation as described in Materials and Methods. The LPL protein of each fraction was resolved by SDS-PAGE and immunoblotted. Arrows indicate the fractions that contain sedimentation markers, BSA (4.1 S) and yeast alcohol dehydrogenase (7.6 S), respectively.

containing lipoproteins (Carrero *et al.*, 1996). Plasma lipoprotein patterns in humans and rats were compared in order to investigate the mechanism of different fates of free LPL in pre-heparin plasma. Plasma lipoproteins were separated by NaBr density gradient ultracentrifugation. As shown in Fig. 5, VLDL, LDL and HDL could be clearly separated in human plasma. VLDL appeared at the topmost fractions. LDL and HDL were detected around the 6th and 13th fractions, respectively. Plasma proteins remained at the bottom and were found after the 20th fraction. But in rat plasma, only VLDL and the broad peak of HDL were identified. A smaller amount of the LDL fraction was observed.

Discussion

LPL is produced and secreted by parenchymal cells in extrahepatic tissues. The production of active LPL by cells involves synthesis and N-linked glycosylation, dimerization of subunits, development of a high affinity for heparin, and secretion (Masuno *et al.*, 1991; Braun and Severson, 1992; Park, *et al.*, 1995). Secreted LPL was transferred to the capillary endothelium by an unknown mechanism (Braun and Severson, 1992). It attached to the luminal surface of vascular endothelial cells via an interaction with the membrane-bound heparan sulfate (Saxena, *et al.*, 1991).

LPL activity was not detected in normal plasma. The injection of heparin caused an increase of both plasma LPL activity and protein. The LPL released by heparin was an active dimer, indicating that functional LPL attached to the endothelium was released into plasma. The activity of the released LPL reached its peak at 30 min, and returned to the baseline value in 4 h.

In spite of no detectable LPL activity, a significant amount of the LPL protein was observed in the immunoblotting of the LPL protein of normal plasma. It has been reported that LPL is continuously dissociated from the endothelial cells under physiological conditions. It also keeps equilibrium with newly synthesized LPL in parenchymal cells (Bagby, 1983).

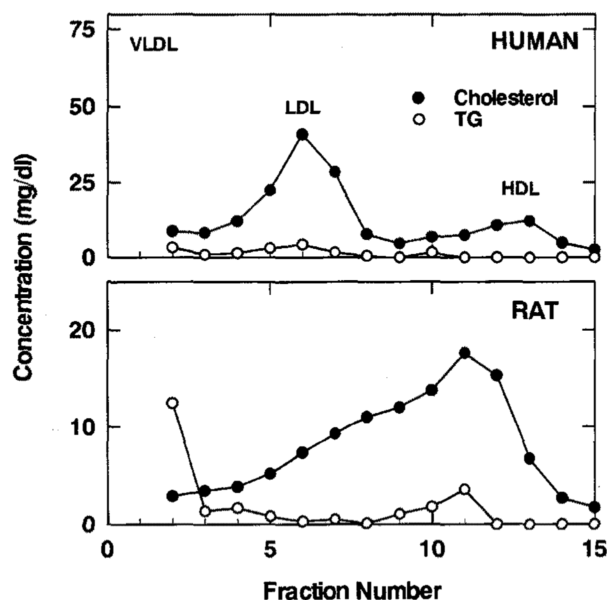

Fig. 5. NaBr density gradient ultracentrifugation profiles of normal human and rat plasma lipoproteins. Lipoproteins of normal human and rat plasma were separated by NaBr density gradient ultracentrifugation, described under Materials and Methods. The triglyceride and cholesterol contents of each fraction were determined.

However, little is known about the degradative pathway of the released LPL protein in the plasma.

The inactive LPL protein in pre-heparin human plasma was detected at the dimeric fractions in sucrose density gradient ultracentrifugation. Active LPL is known as a homodimer (Garfinkel, *et al.*, 1983). Dissociation of the dimer to the monomeric form renders the lipase inactive (Osborne, *et al.*, 1985). Generally, the activity and dimerization were treated as a set. However, our finding of the inactive, but dimeric form of LPL in normal human plasma indicates that dimerization can be an independent entity from the catalytic activity of LPL. The presence of the inactive, but dimeric form of the LPL protein in carbonyl cyanide m-chlorophenylhydrazone-treated 3T3-L1 adipocytes also supports these findings (Park, *et al.*, 1997).

In rats, most of the LPL proteins in post-heparin plasma were active and dimeric as in human post-heparin plasma. But in pre-heparin plasma, the LPL protein was inactive, and a great fraction of the LPL protein is present as an aggregate. Park *et al.* (Park, *et al.*, 1995) already reported that the inactive, low heparin affinity form of LPL in tunicamycin- or castanospermine-treated 3T3-L1 adipocytes was present as an aggregate, not as a monomer. These results suggest that the degradative pathway of the released LPL may be different between humans and rats.

A free form of LPL, released from the endothelial cells, was reported to bind with apo B-containing, cholesterol-rich lipoprotein of the LDL fractions (Vilella, *et al.*, 1993; Carrero, *et al.*, 1996). To investigate the mechanism for the different degradative pathways of the released LPL in rats and humans, plasma lipoproteins were separated by NaBr ultracentrifugation, and their patterns were compared. In human plasma, VLDL, LDL and HDL could be clearly separated under these conditions. However, only VLDL, and the broad peak of HDL, were identified in rat plasma. A smaller amount of the LDL fraction was observed in rat plasma.

HDL in a healthy, normolipidemic man typically represents ~30-32% of the total lipoproteins and LDL preponderate (~50%). Any animal, in which HDL accounts for 50% or more of the total d<1.21 g/ml substances, has been considered as an "HDL mammal" with a profile distinct from that of urban man. These include rodents (mice and rats) and carnivores (dogs, bears, and cats). Humans are considered "LDL mammals", along with guinea pigs and rabbits (Chapman, 1986).

There are reports that LPL may enhance the cholesterol uptake of the arterial wall cells *in vitro* by forming the bridge between plasma lipoproteins and cell surface proteoglycan (Ji *et al.*, 1993; Seo *et al.*, 2000). The presence of a significant amount of the inactive LPL protein in normal plasma suggests that the LPL-mediated LDL uptake may play an important role *in vivo*. The different pathways of LPL degradation among the species might affect the LDL uptake and progress of atherosclerosis.

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