Mice Expressing Only Covalent Dimeric Heparin Binding-deficient Lipoprotein Lipase

MUSCLES INEFFICIENTLY SECRETE DIMERIC ENZYME*†

Received for publication, September 24, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M310559200

E. Peer Lutz‡§, Yuko Kakot§, Hiroaki Yagyu§, Joerg Heeren¶, Steven Marks‡, Thamrah Wright‡, Kristan Melford†, Osnat Ben-Zeev**, Herbert Radner‡, Martin Merkels, Andre Bensadoun, Howard Wong**, and Ira J. Goldberg‡§§

From the ‡Department of Medicine, Columbia University, New York, New York 10032, the §Department of Medicine, University Hospital Hamburg-Eppendorf, and the ¶Department of Medical Biochemistry and Molecular Biology, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Germany, the **Division of Nutritional Sciences, Cornell University, Ithaca, New York 14853, the ***Lipid Research Laboratory, West Los Angeles Veterans Affairs Medical Center, Department of Medicine, University of California, Los Angeles, California 90073, and the ¶¶Institute of Pathology, Johannes Gutenberg University, 55101 Mainz, Germany

Lipoprotein lipase (LpL) hydrolyzes triglycerides of circulating lipoproteins while bound as homodimers to endothelial cell surface heparan sulfate proteoglycans. This primarily occurs in the capillary beds of muscle and adipose tissue. By creating a mouse line that expresses covalent dimers of heparin-binding deficient LpL (hLpL<sub>HBM-Dimer</sub>) in muscle, we confirmed in vivo that linking two LpL monomers in a head to tail configuration creates a functional LpL. The hLpL<sub>HBM-Dimer</sub> transgene produced abundant activity and protein in muscle, and the LpL was the expected size of a dimer (∼110 kDa). Unlike the heparin-binding mutant monomer, hLpL<sub>HBM-Dimer</sub> had the same stability as nonmutated LpL. The hLpL<sub>HBM-Dimer</sub> transgene prevented the neonatal demise of LpL knockout mice; however, these mice were hypertriglyceridemic. Postheparin plasma LpL activity was lower than expected with the robust expression in muscle and was no longer covalently linked. Studies in transfected cells showed that Chinese hamster lung cells, but not COS cells, also degraded tandem repeated LpL into monomers. Thus, although muscle can synthesize tethered, dimeric LpL, efficient production of this enzyme leading to secretion, and physiological function appears to favor secretion of a noncovalent dimer composed of monomeric subunits.

Triglycerides (TG)<sup>1</sup> in circulating lipoproteins are hydrolyzed by lipoprotein lipase (LpL). This enzyme is synthesized primarily in adipose tissue and muscle, and is transferred to the luminal surface of endothelial cells, and associates with heparan sulfate proteoglycans. This association with heparan sulfate proteoglycan is thought to localize LpL and direct liberated free fatty acids to the tissues where LpL is expressed (1). By creating transgenic mice expressing a heparin-binding mutant of human LpL (hLpL<sub>HBM</sub>), we showed that mice expressing this protein have an alteration in tissue delivery of fatty acids derived from lipoprotein TG (2).

The association of a protein with heparin is thought to have several biochemical implications. In the case of LpL, heparan sulfate proteoglycan association fixes LpL to the endothelial surface (3). In addition, heparin stabilizes LpL activity (4). A similar process occurs for other heparin-binding proteins such as basic fibroblast growth factor. Like basic fibroblast growth factor, LpL is a homodimeric or oligomeric molecule, and its dissociation into monomers is thought to be part of its regulation (5, 6). High affinity heparin binding could stabilize LpL by maintaining LpL secondary and tertiary structure, thereby modulating the dissociation of LpL into monomeric units.

In vitro studies have shown that active LpL can be produced when a short hinge is used to create a head to tail dimer of two monomeric subunits (7). Using this technique, we created a tandem repeat of hLpL<sub>HBM</sub> and expressed this protein in transgenic mice. This allowed us to study the role of heparin affinity in LpL stability and the importance of monomer to dimer assembly in the secretion of LpL from muscles.

MATERIALS AND METHODS

Generation of Transgenic Mice—Based on human heparin-binding site mutated LpL, hLpL<sub>HBM</sub> (2), a minigene was created that encodes for muscle-specific expression of two covalently linked hLpL<sub>HBM</sub> monomers in a head to tail configuration. First a PCR over the hLpL<sub>HBM</sub> minigene Bluescript vector (pBhLpL<sub>HBM</sub>) was performed utilizing VENT polymerase (New England Biolabs, Beverly, MA), sense primer 1 (ctc cca cga gcg ctc gtg gga) and antisense primer 2 (ttg ggg cga gcg ctc ttc cca), and amplified by 25 cycles. A second PCR was performed utilizing the sense primer 1 and antisense primer 3 (gtc ctc cta gct cat cta) and amplified by 20 cycles.

**To whom correspondence should be addressed: Dept. of Medicine, Columbia University, 630 West 168th St., New York, NY 10032. Tel.: 212-305-3678; Fax: 212-305-5384; E-mail: ijg3@columbia.edu.

† This work was supported by Grants HL45095 (to I. J. G.), HL014990 (to A. B.), and HL028481 (to H. W.) from the National Institutes of Health and Sachbeihilfe LU855/2-1 (to E. P. L.) and Me1507/2-1 (to M. M.) from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡§§ To whom correspondence should be addressed: Dept. of Medicine, Columbia University, 630 West 168th St., New York, NY 10032. Tel.: 212-305-3678; Fax: 212-305-5384; E-mail: ijg3@columbia.edu.

¶ The abbreviations used are: TG, triglyceride(s); LpL, lipoprotein lipase; hLpL, human LpL; hLpL<sub>HBM</sub>, heparin-binding mutant hLpL; hLpL<sub>HBM-Dimer</sub>, covalent dimeric heparin-binding mutant hLpL; LpL<sub>0</sub>, covalent native LpL tandem repeat; CHL, Chinese hamster lung; LpL<sub>D</sub>, homozygous LpL knockout mice; PHP, postheparin plasma; ELISA, enzyme-linked immunosorbent assay.
LpL protein (gcc at position 256 in the hLpL cDNA open reading frame). By using the NotI and Eco47III sites, this PCR product was inserted in pBlpLHBM to produce the intermediate plasmid pBl2. This generated a fragment of the linker and the linker region coupled to the hLpLHBM minigene downstream from the Eco47III site including the heparin binding mutations and the 3′-untranslated region. Finally, the linker Lpl fragment of pBl2 was inserted in pBl1 by using the XhoI and EcoRV restriction enzyme sites. The resulting hLpLHBM minigene (Fig. 1) was sequenced, purified, and used for production of transgenic mice. Founder animals were crossed with heterozygote LpL knockout mice that had been backcrossed seven times to C5BL/6. Pups heterozygote for both hLpLHBM-Dimer and the LpL knockout allele were crossed again with heterozygous LpL knockout mice. 12.5% of the subsequent pups were homozygous for the LpL knockout allele (LpL0) and heterozygote for the hLpLHBM-Dimer transgene. Finally, the linker LpL fragment of pBi2 was inserted in pBi1 by phage P1 and P3, and inserted into the pcDNA3 expression vector (Invitrogen). COS-7 and Chinese hamster lung (CHL) cells were transfected with a plasmid DNA mixture that contained the hLpLHBM minigene (2) and nonmutated hLpL (8) were bred in the same manner.

Genotyping of Transgenic Mice—Tail tip DNA was screened by PCR. The genotype at the mouse LpL locus was analyzed by the 3′ PCR as described. The hLpLHBM-Dimer transgene was detected by oligonucleotide primers in the muscle creatine kinase promoter and the linker region. The hLpLHBM and hLpL transgenes were detected by utilizing the hLpL PCR (9).

Plasma Lipid and Lipoprotein Analysis—The mice were fed a chow diet (4.5% fat, w/w). The plasma samples were collected after 6 h of daytime fasting. Plasma TG and cholesterol were determined with kits (Sigma-Aldrich) in duplicate. For lipoprotein analysis fast performance liquid chromatography analyses of pooled plasma, the samples were performed as described (10). In addition, individual plasma samples (60 μl) were ultrafiltrated twice in a Beckman TLA-100 rotor (Beckman Coulter, Fullerton, CA) as described previously (11).

LpL Mass and Activity Measurements—To obtain postheparin plasma (PHP), fasted mice were bled 5 min after a tail vein injection of 100 units of heparin/kg body weight (Elkins-Sinn, Cherry Hill, NJ). Human and murine LpL protein was measured by ELISA as described previously by Peterson et al. (12). LpL activity was measured by the method described by Hocquette et al. (13). To distinguish lipolysis mediated by human LpL from activity caused by mouse LpL and hepatic lipase, the mouse plasma samples were assayed in the presence of a monoclonal antibody against human LpL (14) and under high salt conditions (final concentration, 1 M NaCl).

LpL Stability—LpL stability was assessed using muscle homogenates of quadriiceps muscles from transgenic mice. 100 mg of wet weight muscle was homogenized in 900 μl of homogenization buffer (25 mM NH4HCl, 5 mM EDTA, 0.8% w/v Triton X-100, 0.01% w/v SDS, 5 units/ml heparin, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 0.017 M TIU/ml aprotinin, pH 8.2) as described (13). After a 30-min centrifugation at 20,000 × g for 4 °C, the supernatants were frozen in aliquots (−70 °C) and then defrosted just prior to stability experiments. To assay LpL stability, the LpL containing muscle homogenates were incubated in a water bath at 37 °C. At the indicated time points the aliquots were frozen and immediately assayed for LpL activity.

Northern and Western Blot Analysis of LpL from Quadriiceps Muscles—For Northern blots, total RNA was prepared from quadriiceps muscles and hearts of 12 week-old hLpLHBM/LpL0 and LpLHBM-Dimer/LpL0 male mice using a kit (TRIZol reagent, Invitrogen). 15 μg of total RNA were applied to a 1% agarose gel and blotted to a Hybond-N+ Nylon Membrane (Amersham Biosciences) using standard techniques. The blot was hybridized with a probe spanning the −500-bp RareII/HindIII fragment from the hLpL minigene and then autoradiographed. To confirm that equal amounts of RNA were loaded to each lane, the blot was probed for glyceraldehyde-3-phosphate dehydrogenase.

For Western blot analyses muscle homogenates of 20 week-old hLpL/LpL0 and hLpLHBM-Dimer/LpL0 male mice were analyzed as described above. To increase sensitivity, the homogenates were partially purified by heparin-Sepharose essentially as described (15, 16). Briefly, 10 μl of resuspended heparin-Sepharose was added to 1 ml of muscle homogenate and incubated for 3 h at 4 °C. The Sepharose was washed with a Tris-buffer containing 0.75 M NaCl. LpL then was eluted with 50 μl of a Tris buffer containing 1.5 M NaCl. SDS-PAGE under reducing conditions with 5 μl of eluates and blotting to nitrocellulose membranes were performed using standard techniques. The membrane was incubated with a polyclonal rabbit anti-bovine LpL antibody (SA1357; 1:2500 dilution) and an anti-rabbit IgG (Sigma-Aldrich; 1:5000 dilution). For SA1357 production, bovine LpL was kindly provided by Dr. G. Olivercrona (Unnea, Sweden). The antibody was produced by Eurogentec (Herstal, Belgium). The bands were visualized by ECL (Amersham Biosciences).

Expression of Dimeric LpL in Cell Culture—A full-length cDNA for a tandem repeat dimeric LpL, LpL™, has been reported (7). The cDNA was purified, digested, and inserted into the pcDNAS expression vector (Invitrogen). COS-7 and Chinese hamster lung (CHL) cells were transfected with the pcDNAS/LpL™ expression vector. After transfection of COS cells, the calcium phosphate-DNA mixture was left overnight on the cells and then removed by a rinse with phosphate-buffered saline. The cells were then treated with 3 ml of 10% (v/v) MeSO in phosphate-buffered saline for 2.5 min. After removal of the Me2SO solution, fresh Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. Co-precipitates of plasmid DNA and CaPO4 were prepared according to the manufacturer’s instructions to mediate cell transfections. The calcium phosphate-DNA mixtures were incubated at room temperature for 30 min prior to addition to 50–60% confluent cells. For transient transfection of COS cells, the calcium phosphate-DNA mixture was left overnight on the cells and then removed by a rinse with phosphate-buffered saline. The cells were then treated with 3 ml of 10% (v/v) MeSO in phosphate-buffered saline for 2.5 min. After removal of the Me2SO solution, fresh Dulbecco’s modified Eagle’s medium supplemented with serum substitute (Nutridoma, Roche Applied Science) and 20 units/ml heparin was added to each dish. The medium was harvested daily for a 3-day period and stored at −80 °C. Stably transfected CHL cells were selected by growth in the presence of Geneticin (G418, 500 μg/ml, Sigma-Aldrich). The surviving colonies were expanded and assayed for lipase activity. The cell clones with the highest levels of lipolytic activity were used for expression in T-225 flasks containing Dulbecco’s modified Eagle’s medium, supplemented with 1% Nutridoma and 10 units/ml heparin. The medium was harvested daily for up to 30 days and stored at −80 °C.

Heparin-Sepharose Chromatography—Affinity chromatography was performed by using a fast performance liquid chromatography system (Amersham Biosciences) with a 1-ml Hi-Trap heparin-Sepharose column. The cell culture supernatants of LpLHBM-Dimer or native LpL expressing cells were loaded to the column. The LpL mass concentrations in the fractions were determined by ELISA as described (17).

Western Blots of PHP and Cell Culture LpL—PHP samples were subjected to sucrose gradient centrifugation and immunoprecipitation exactly as described (18). For Western blot, the samples were mixed with a half-volume of buffer containing 2% SDS, 0.1 x Tris-HCl, pH 6.8, 50% glycerol, 10% β-mercaptoethanol, 0.05% bromphenol blue. The mixtures were placed in boiling water for 5 min prior to loading onto a 7% acrylamide gel. The gels were electroblotted onto nitrocellulose and subsequently blocked for 1 h in Tris-buffered saline containing 5% bovine serum albumin. After blocking the membranes were incubated overnight at 4 °C with a monoclonal antibody that detects human, but not mouse, LpL (5D2, kindly provided by J. Brunzell). The 5D2 epitope is in the C-terminal portion of native LpL (19). Immunoblots were developed with anti-mouse IgG conjugated to biotin. After washing, the blots were incubated with streptavidin conjugated to horseradish peroxidase. Immunoreactive bands were visualized with chemiluminescent reagents (Pierce) and exposure to x-ray film.

Histological Analysis—Quadriiceps muscles were dissected from 6-month-old male hLpLHBM-Dimer/wild type mouse LpL and wild type littermates and fixed in formaldehyde. Histological analyses were performed as described (20).

RESULTS

In Vitro Expression of hLpLHBM-Dimer and Heparin Binding—Medium obtained from cells expressing native hLpL or
hLpL/HBM-Dimer was chromatographed. Elution of native LpL from heparin-Sepharose leads to two peaks: one peak eluting at >1 M NaCl that contains active dimeric enzyme (Fig. 2) and, commonly, a second peak that elutes with −0.75 M NaCl and is not associated with activity. Presumably this second peak is inactive monomeric or misfolded protein. LpL/HBM primarily is found in the latter peak, as has been previously reported (2). LpL/HBM-Dimer protein eluted with a single peak at −0.9 M NaCl. Thus, the construct produced an enzyme whose heparin affinity was lower than that of nonmutated LpL but greater than that of monomeric LpL.

Dimer Expression in Muscle—Our studies of the transgenic mice focused on the actions of the hLpL/HBM-Dimer transgene on the LpL-deficient background because this allowed measurements of LpL without interference from the native enzyme. Northern blots were performed to verify that the hLpL/HBM-Dimer construct was expressed in muscles from mice. As shown in Fig. 3A, the mRNA of the hLpL/HBM-Dimer/LpL0 mice expressing the dimeric construct led to a larger transcript than that found in hLpL/HBM/LpL0 or hLpL/LpL0 mice. The line of mice that was bred also had much greater expression of the transgene. Moreover, these mice produced a larger LpL protein of ~110 kDa in muscle tissue (Fig. 3B). This is the expected size of the dimeric protein.

Enzyme Activity and Protein in Muscles—Homogenates of left quadriceps and hearts from mice expressing hLpL, hLpL/HBM, and hLpL/HBM-Dimer constructs on the LpL0 background (n = 3 each line) were assayed for LpL activity and mass. The average activity found in the hLpL/HBM-Dimer/LpL0 mice (Fig. 4A) was similar to that in the hLpL/HBM transgenics and greater that found in mice expressing homomutant human LpL (8).

LpL protein was assayed in muscle homogenates from these three lines of mice. In concert with the RNA and activity data, hLpL/HBM-Dimer muscles contained ~1.5 times the LpL protein of hLpL/HBM and more than 10 times as much protein as the line of LpL-expressing mice (Fig. 4B). The specific activities in muscle tissues were 0.7 for hLpL/HBM-Dimer versus 1.1 for hLpL/HBM and 2.8 μmol of free fatty acid/μg/h for hLpL-expressing mice on LpL0 background (Fig. 4C).

Muscle Histology—Histological analysis of quadriceps muscles from hLpL/HBM-Dimer transgenic mice revealed no major pathological changes. A slight increase in lipocytes in the endomysium and subsarcolemmal enhancement of periodic acid Schiff-positive material, minor histopathologic changes, were noted in hLpL/HBM-Dimer muscle. Most significantly, no lipolysis-induced myopathy like that in mice overexpressing hLpL in muscles (21) was seen in these animals.

Stability of LpL Activity from Muscles of hLpL/HBM-Dimer Mice—To determine whether the dimeric configuration altered the stability of the mutated LpL, muscle homogenates from each line of mice were incubated at 37 °C, and the activity was assessed over time (n = 3 each line). A slow decay of activity was found in muscles expressing both hLpL and hLpL/HBM-Dimer (Fig. 4D). In contrast, hLpL/HBM was less stable. Therefore, the instability associated with the mutation in heparin binding was corrected by creating the tethered dimer.
Lipids and Lipoproteins in \textit{hLpL} HBM-Dimer Transgenic Mice—Plasma lipids and lipoproteins were assessed in the \textit{hLpL} HBM-Dimer mice and were compared with the other transgenic lines. Although the \textit{hLpL} HBM-Dimer transgene rescued the LpL knockout mice, plasma TG in these mice was more than twice that found in animals expressing either the native \textit{hLpL} or the \textit{hLpL} HBM transgene (Table I). This was due to an increase in very low density lipoprotein TG as noted both by ultracentrifugation (Table I) and by gel filtration chromatography (Fig. 5). Plasma cholesterol was not different between these mice. Thus, despite the robust expression of the \textit{hLpL} HBM-Dimer transgene, these mice have higher TG. This suggested that either the dimer construct was less active in the plasma or that the amount of this LpL in the PHP did not parallel the muscle expression (Fig. 4).

\textbf{PHP LpL Activity and Mass in PHP}—The PHP LpL activities and masses of the three lines of mice are shown in Fig. 6. Surprisingly, \textit{hLpL} HBM-Dimer/LpL0 mice had less LpL activity than mice expressing either \textit{hLpL} HBM or \textit{hLpL} despite the greater expression of this protein in the muscle. The specific activities in PHP were 0.7 for \textit{hLpL} HBM-Dimer versus 1.5 for \textit{hLpL} HBM and 4.5 \textmu mol of free fatty acid/\textmu g/h for \textit{hLpL} expressing mice on LpL0 background. Thus, activity of the dimeric protein was unexpectedly low, which is consistent with the increased TG in these mice.

\textbf{PHP LpL Western Blots}—Because muscle, but not PHP, from \textit{hLpL} HBM-Dimer mice contained more LpL activity, we questioned whether the dimeric protein was altered either prior to or after its secretion. To test this the protein was first isolated using sucrose gradients; active LpL sedimented as a dimeric protein in all three samples (data not shown). Surprisingly, Western blots of PHP LpL from the \textit{hLpL} HBM-Dimer transgenic mice produced a band the same size as bovine LpL and wild type human LpL, \textit{i.e.}, PHP did not contain covalently linked LpL dimers (Fig. 7). Thus, either the muscle did not secrete the covalent dimer LpL or the protein was degraded after its secretion.

\textbf{Expression of Dimeric LpL in Vitro}—To test the possibility that the LpL dimer construct is “clipped” by cells, we expressed dimeric LpL protein in two different cell lines. To rule out the possibility that clipping of the dimeric construct was due to the C-terminal heparin-binding mutations, we utilized nonmutated covalent dimeric LpL for cell culture expression (LpLTR) (7). LpLTR was expressed in CHL cells and in COS cells. Western blots of medium from COS cells showed a dimeric-sized LpL protein (Fig. 8A). However, when the same construct was expressed in CHL cells, the medium contained only monomeric LpL. Thus, some cells and presumably tissues like muscle are unable to secrete dimeric forms of LpL. Western blot of cell fractions of CHL and COS cells expressing LpLTR showed that CHL cells contained intracellular products of the dimer of \textapprox 85, 66, and 63 kDa (Fig. 8B).

To further investigate where clipping of the covalent dimeric protein occurs, we looked for protease activity of cell culture supernatants of CHL cells. We mixed media of nontransfected CHL cells with supernatants of COS cells expressing LpLTR and incubated the samples for 1 h at room temperature. Then

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{\textbf{LpL activity, mass, and stability of \textit{hLpL} HBM-Dimer.} Homogenates of quadriceps muscles of transgenic mice on the LpL knockout background were prepared as described. \textbf{A}, LpL activity in muscles of \textit{hLpL} HBM-Dimer/LpL0 mice was as high as in \textit{hLpL} HBM expressing muscles but about three times higher than in \textit{hLpL} expressing muscles. \textbf{B}, immunoreactive LpL mass was the highest in \textit{hLpL} HBM-Dimer expressing muscles. \textbf{C}, specific LpL activity was the lowest in \textit{hLpL} HBM-Dimer expressing muscles. \textbf{D}, the activity stability was measured by incubating muscle homogenates at 37 °C and assaying aliquots for LpL activity at the indicated time points. LpL activity in \textit{hLpL} HBM-Dimer muscles (closed circles) was comparable with that of nonmutated \textit{hLpL} (open triangles), whereas \textit{hLpL} HBM (open squares) showed reduced stability.}
\end{figure}
we assayed for lipase activity and analyzed the mixes by LpL Western blot. Nontransfected CHL medium did not affect lipase activity of transfected COS cell media, and no additional monomeric sized LpL band was detected by immunoblot, i.e. no proteolysis occurred. Also when medium from CHL cells that were stably transfected with LPLTR was mixed with the LpLTR-COS medium, the activity and size in Western blot analysis of the covalent dimeric protein remained unaffected. These results are in concert with additional experiments in which protease inhibitors like benzamidine or phenylmethylsulfonyl fluoride did not inhibit the clipping of LpLTR expressed by CHL cells. In conclusion there is no evidence for the involvement of an extracellular protease in this process, at least in the in vitro setting.

DISCUSSION

Several processes are thought to be central to the regulation of LpL activity in vivo: 1) LpL is expressed as a monomeric
A protein and then assembled into a dimer to allow its physiological action; this appears to be a complex process (20). 2) LpL actions are limited by LpL dissociation from heparan sulfate proteoglycan on capillaries and its clearance in the liver (21). 3) The dissociation of the dimeric protein into subunits in the bloodstream then prevents further lipolysis from occurring. In a previous study we showed that a mutation in a heparin-binding region of LpL led to instability of the dimeric LpL complex. Large amounts of this mutated LpL were found in the pre-heparin plasma; however, we were unable to determine whether defective heparin binding per se or conversion of active LpL to monomers was the primary defect leading to enzyme instability (2). By creating a hinged dimer of heparin-binding defective LpL, we hoped to better understand the roles of heparin association and dimer to monomer interconversion in LpL biology. Our studies showed the following: 1) hLpL'HBM-Dimer associated with heparin-Sepharose; however, it was dissociated with \(-0.9 \times \text{NaCl}\). Thus, its affinity for heparin was reduced compared with native LpL. 2) hLpL'HBM-Dimer was expressed in high levels in muscles of transgenic mice, and the dimeric mRNA and protein were demonstrated. 3) Tissues from these mice had LpL activity with greater stability than that found with hLpL'HBM. 4) Although the hLpL'HBM-Dimer transgene rescued LpL knockout mice, the mice were hyperlipidemic. This suggested that the LpL was less active. 5) Despite the robust expression of this transgene in muscle, plasma LpL activity was less than in transgenic mice expressing hLpL and hLpL'HBM, and the specific activity of the LpL was the lowest of the three lines of mice. Moreover, no covalently linked dimeric protein was found in the PHP of the hLpL'HBM-Dimer transgenic mice. Thus, muscle inefficiently produces dimeric LpL, and conversion of dimer to monomers must occur either prior to or after secretion. 6) Similar clipping of the dimer occurred in CHL cells but not COS cells. Therefore the ability to secrete dimeric LpL is limited to only some types of cells. 7) Monomeric sized LpL fragments were found within these cells. Thus, it is likely that the clipping occurs intracellularly.

In our previous studies of mutated LpL, alteration of C-terminal basic amino acids led to two effects: 1) reduced association of the protein with heparin and 2) reduced stability of the protein. By introducing this mutation into an obligate dimer, we hoped to dissect the roles of each of the processes in LpL biology. We first showed that hLpL'HBM-Dimer was defective in heparin binding. Like the active forms of hLpL'HBM, this protein eluted from a heparin affinity column at 0.8–0.9 \( \times \text{NaCl} \) rather than \( >1.2 \times \text{NaCl} \), the elution of native LpL. When LpL'HBM-Dimer was expressed within muscle, unlike hLpL'HBM, the dimeric protein had normal stability. Thus, tethering restored stability and suggested that defective heparin-association alone likely led to more rapid monomerization, which in turn reduced LpL activity.

Several observations suggested that the dimeric LpL was processed aberrantly in vivo. Despite the robust expression of the transgene and large amounts of activity in the muscle homogenates, hLpL'HBM-Dimer mice had higher TG than mice expressing hLpL and even hLpL'HBM. Moreover, the specific activity of PHP hLpL'HBM-Dimer was lowest in these mice. This led us to question whether hLpL'HBM-Dimer was altered in some manner. Western blot analysis of PHP demonstrated only monomeric LpL-sized enzyme. Thus, the protein was inefficiently secreted because the PHP did not have more protein despite more robust muscle expression, and the dimer was clipped into monomeric units either prior to or after secretion from the muscle.

Because culture of the myocytes from the transgenic mice was not a viable option, we studied other cells to determine whether they also degraded dimeric LpL into monomers. Moreover, we elected to study this effect using LpL that was not mutated so we could assess whether the clipping was due to the dimeric construct and not another mutation in the LpL. LpL'TM was efficiently produced and secreted from COS but not from CHL cells. This surprising observation allowed us to understand the observations that we had in the hLpL'HBM-Dimer mice. Skeletal muscles, like CHL cells, appear to be unable to secrete active dimeric LpL and clip the protein, which leads to only monomeric forms within the PHP.

Several experiments were performed to determine the site of

Fig. 7. Western blot of PHP from different mouse lines. PHP of mice expressing hLpL'HBM-Dimer, hLpL'HBM, and hLpL on the LpL0 background was subjected to sucrose gradient centrifugation. Aliquots of the fractions with LpL activity were pooled, and immunoprecipitation and Western blot were performed as described under “Materials and Methods.” Lane 1, controls: bovine LpL (55 kDa) and cell culture supernatants of COS cells expressing LpL'TM. Lane 2, PHP of hLpL'HBM-Dimer LpLo. Lane 3, PHP of hLpL'HBM/LpLo. Lane 4, PHP of hLpL/LpLo.

Fig. 8. Western blot of media and cell fractions from CHL and COS cells expressing LpL'TM. Medium and cell fractions from either stably transformed CHL cells (lane 1) or transiently transfected COS cells (lane 2) was analyzed by SDS-PAGE, electrophoresed, and probed with monoclonal antibody to native LpL. The migration position of prestained molecular mass standards (in kDa) is indicated on the left. A, Western blot of LpL'TM-transfected medium from CHL cells demonstrated an immunoreactive band at \(-50 \text{kDa} \); that in COS cells was at \(-100 \text{kDa} \). B, Western blot of CHL cells (lane 1) and COS cells (lane 2) expressing LpL'TM, demonstrating three intracellular bands of \(-85, 66, \) and \(-63 \text{kDa} \) in CHL cells and a single band of dimer size (\(-100 \text{kDa} \)) in COS cells.
dimer proteolysis. Most interesting, we found that CHL but not COS cells secreted clipped dimer. Thus, the ability to produce dimeric forms of LpL was cell-specific and conceivably might be tissue-specific in vivo. We attempted to define where the proteolysis occurred. Medium from CHL cells did not appear to have a protease, and lysosomes inhibitors did not prevent cleavage of dimer. However, intracellular cleavage of dimer occurred in the CHO cells. Thus, we postulate that dimeric protein is clipped within cells prior to its secretion.

LpL is not unique in its requirement for dimeric association to optimize its biologic activity. Moreover, a number of dimeric molecules associate with heparin. Our data suggest that heparin maintenance of the dimer, rather than heparin association itself, stabilizes LpL because activity in muscles expressing mutated dimers was as stable as nonmutated LpL.

Because the requirements for dimerization do not appear to be the most efficient method to produce an important metabolic enzyme, there have been a number of hypotheses to explain this mode of synthesis. One obvious reason might be to allow for inactivation of the enzyme, thus preventing excessive local lipolysis and accumulation of toxic levels of reactive lipid products. Our data suggest an entirely new paradigm for monomeric protein production. As we found, in vivo, myocytes are incapable of efficient production of tethered dimeric LpL. In contrast, it is well established that LpL monomers assemble as dimers prior to their secretion from several cells types (20). Either myocytes are different, or the requirements for secretion of the tethered dimer differ from that of the assembled homodimer. Although our studies are limited to LpL and its production in muscle, we hypothesize that the secretion pathways of other dimeric heparin-binding proteins are cell-specific. Maybe an intracellular or less likely an extracellular protease, in some cells, specifically prevents production of tethered dimeric proteins.

**REFERENCES**

1. Merkel, M., Eckel, R. H., and Goldberg, I. J. (2002) *J. Lipid Res.* 43, 1997–2006
2. Lutz, E. P., Merkel, M., Kako, Y., Melford, K., Radner, H., Breslow, J. L., Bensadoun, A., and Goldberg, I. J. (2001) *J. Clin. Invest.* 107, 1183–1192
3. Shimada, K., Gill, P. J., Silbert, J. E., Douglas, W. H., and Fanburg, B. L. (1981) *J. Clin. Invest.* 68, 995–1002
4. Lookene, A., Chevreuil, O., Ostergaard, P., and Olivecrona, G. (1996) *Biochemistry* 35, 12155–12163
5. Vlodavsky, I., Zhao, H. Q., Medalion, B., Danagher, P., and Ron, D. (1996) *Cancer Metastasis Rev.* 15, 177–186
6. Berto, M., Olivecrona, G., and Olivecrona, T. (1996) *Biochem. J.* 313, 893–898
7. Wong, H., Yang, D., Hill, J. S., Davis, R. C., Nika, J., and Schotz, M. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 5594–5598
8. Levak-Frank, S., Radner, H., Walsh, A., Stollberger, R., Knipping, G., Hoefler, G., Sattler, W., Weinstock, P. H., Breslow, J. L., and Zechezner, R. (1995) *J. Clin. Invest.* 96, 976–986
9. Levak-Frank, S., Weinstock, P. H., Hayek, T., Verdeny, R., Hofmann, W., Ramakrishnan, R., Sattler, W., Breslow, J. L., and Zecheze, R. (1997) *J. Biol. Chem.* 272, 17182–17191
10. Kako, Y., Huang, L. S., Yang, J., Kutopolis, T., Ramakrishnan, R., and Goldberg, I. J. (1999) *J. Lipid Res.* 40, 2185–2194
11. Havel, R. J., Eder, H. A., and Bragden, J. H. (1955) *J. Clin. Invest.* 34, 1345–1353
12. Peterson, J., Fujimoto, W. Y., and Brunzell, J. D. (1992) *J. Lipid Res.* 33, 1165–1170
13. Hocquette, J. F., Graulet, B., and Olivecrona, T. (1998) *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 121, 201–212
14. Goldberg, I. J., Paterniti, J. R., Jr., France, D. S., Martinelli, G., and Cornicelli, J. A. (1986) *Biochim. Biophys. Acta* 878, 168–176
15. Zechner, R. (1990) *Biochim. Biophys. Acta* 1044, 25–25
16. Ben-Avram, C. M., Ben-Zeev, O., Lee, T. D., Haaga, K., Shively, J. E., Goets, J., Pedersen, M. E., Reeve, J. R., Jr., and Schotz, M. C. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 4185–4189
17. Sendak, R. A., and Bensadoun, A. (1998) *J. Lipid Res.* 39, 1310–1315
18. Ben-Zeev, O., and Doolittle, M. H. (1999) *Methods Mol. Biol.* 109, 257–266
19. Chang, S. F., Reich, B., Brunzell, J. D., and Will, H. (1998) *J. Lipid Res.* 39, 2350–2359
20. Ben-Zeev, O., Ma, H. Z., and Doolittle, M. H. (2002) *J. Biol. Chem.* 277, 10727–10738
21. Wallinder, L., Peterson, J., Olivecrona, T., and Bengtsson-Olivecrona, G. (1984) *Biochim. Biophys. Acta* 795, 513–524