Lipoprotein Lipase Is Produced by Cardiac Myocytes Rather Than Interstitial Cells in Human Myocardium

Kevin D. O'Brien, Marina Ferguson, David Gordon, Samir S. Deeb, Alan Chait

Abstract Lipoprotein lipase (LPL) may play an important role in myocardial metabolism by releasing free fatty acids from triglycerides for oxidation by myocytes. However, studies in species other than humans have differed in their conclusions as to whether LPL is produced by cardiac myocytes or interstitial cells. The location and source of LPL in human myocardium were determined on formalin-fixed samples from 25 cardiomypathy patients and seven control patients. LPL mRNA was detected immunohistochemically on cardiac myocytes and adipocytes, but LPL mRNA was not detected in interstitial cells. Quantitative in situ hybridization failed to reveal correlations between LPL mRNA levels and New York Heart Association functional class, left ventricular ejection fraction, or β-adrenergic agonist therapy. Also, quantitative in situ hybridization demonstrated apparently linear loss of detectable myocardial mRNA after onset of ischemia, with a disappearance half-time of ≈26 hours. In summary, LPL is produced primarily by cardiac myocytes rather than by interstitial cells in human myocardium. Furthermore, LPL protein is present on cells with and without detectable LPL mRNA, suggesting that LPL is translocated from sites of synthesis to sites of utilization. (Arterioscler Thromb. 1994;14:1445-1451.)

Key Words • immunocytochemistry • β-adrenergic agonists • immunohistochemistry • energy metabolism • in situ hybridization

Lipoprotein lipase (LPL) (EC 3.1.1.34) hydrolyzes the core triglycerides of chylomicrons and very-low-density lipoprotein (VLDL) to free fatty acids and monoglycerides. The free fatty acids thus liberated from triglycerides by LPL can then be used for anabolic activities, such as fat storage in white adipose tissue, milk synthesis in the lactating breast, and surfactant synthesis in the lung, or for catabolic activities, such as thermogenesis in brown adipose tissue and oxidative metabolism in muscle. Therefore, LPL plays central roles in the maturation of lipoproteins, in the synthesis of important lipid-containing compounds, and in energy metabolism. In the heart, fatty acid metabolism is particularly important because fatty acid oxidation is the major source of energy for the myocardium.

LPL activity has been detected in the myocardium from several species3-6 and is, for the most part, regulated in parallel with skeletal muscle activity in response to various physiological states, such as fasting, feeding, and exercise.1,3-5 Short-term treatment with adenylate cyclase activators, such as cholera toxin, has been shown to increase myocardial LPL activity,5 and LPL activity in the heart has been found to be associated both with cardiac myocytes and with the luminal surface of the endothelium.3 LPL may play an important role in myocardial energy metabolism by liberating fatty acids from circulating triglycerides for oxidation,3 but controversy exists as to whether cardiac myocytes or interstitial cells are responsible for LPL production in the myocardium. LPL mRNA has been detected in guinea pig cardiac myocytes by in situ hybridization,7 and 78% of the LPL protein in mouse heart has been shown by immunohistochemistry to be associated with cardiac myocytes.8 However, in rats, studies that used in situ hybridization have demonstrated that LPL mRNA is expressed primarily by interstitial cells,4 as has been reported for cells cultured from rat myocardium.9 This latter finding is surprising, because the 5'-flanking region of the human LPL gene has been shown to contain three muscle-specific motifs as well as calcium- and cAMP-responsive elements,10 suggesting that LPL expression by cardiac myocytes is likely. However, vascular pericytes, which comprise a portion of myocardial interstitial cells, have some characteristics of muscle cells, including expression of muscle actin,11 and might be likely to express LPL as well. Therefore, the present study was undertaken to determine which cell types in the human myocardium produce LPL, as determined by in situ hybridization. Results indicate that cardiac myocytes are the major source of LPL in human myocardium, although LPL protein can be detected by immunohistochemistry in association with several cell types.

Methods

Myocardial Tissue

Human myocardial segments were obtained at the University of Washington Medical Center from 25 patients undergo-
ing orthotopic cardiac transplantation for severe (New York Heart Association [NYHA] functional class III [symptoms with moderate exertion] or IV [symptoms at rest]) cardiomyopathy. Control myocardial samples from seven patients who were donors of solid organs other than the heart were provided by the Northwest Tissue Center. The seven organ donors were excluded as heart donors primarily for logistical reasons, and none had either clinically evident or echocardiographically demonstrable myocardial dysfunction at the time of death. Ischemic times were recorded for each sample and were defined as the time from onset of cardiac asystole to placement of tissue into 10% neutral-buffered formalin (NBF). Myocardial tissue was placed in NBF within 2 hours of the onset of ischemia in all 25 cardiomyopathy patients. For three cardiomyopathy patients, additional samples were held at room temperature and then placed in NBF at intervals of 1, 3, 6, 12, 18, 24, 30, and 40 hours after onset of ischemia.

Ischemic times for the seven organ donors ranged from 9 to 38.5 hours. All 32 samples were considered as fasted-state samples because all 25 cardiomyopathy patients were fasted in anticipation of surgery and all seven donors had been unconscious and unresponsive before they were declared brain-dead and at least 12 hours had elapsed between the declaration of brain death and organ recovery surgery. Consent for the use of tissues in this research study was obtained in all instances, either from the patients themselves or from their legal next of kin, and the study was approved by the Human Subjects Review Committee of the University of Washington.

Immunohistochemistry

Single-label immunoperoxidase staining of tissues was performed as described previously, using the rabbit polyclonal antiserum LPL-5780 and the mouse monoclonal antibody S9F, both of which detect LPL protein on formalin-fixed human tissue. Monoclonal antibody S9F was partially purified from ascites by an ammonium sulfate/caprylic acid precipitation. LPL-5780 was used at a titer of 1:500 and S9F at a titer of 1:25. Briefly, tissue sections were deparaffinized with xylene, rehydrated with graded alcohols, and washed with phosphate-buffered saline (PBS). The slides were incubated for 30 minutes with the primary antiserum or antibody and washed with PBS; afterward, an anti-rabbit or anti-mouse biotin-labeled secondary antibody was applied (30 minutes), followed by an avidin-biotinylated-peroxidase conjugate (30 minutes). Standard peroxidase substrate (3,3'-diaminobenzidine; Sigma Chemical Co) plus nickel chloride was added to yield a black reaction product. The slides were then counterstained with methyl green.

Riboprobe Preparation

A 1.4-kb human LPL cDNA fragment corresponding to bp 271 to 1630 of the LPL coding sequence was cloned into the vector pGem 1 (Promega) was transcribed into an antisense riboprobe with reagents obtained from Promega except for 35S-UTP, which was obtained from New England Nuclear. A sense riboprobe also was synthesized from a 0.8-kb fragment of the LPL coding sequence corresponding to bp 271 to 1036. The transcription reaction mixture contained 1 µg LPL cDNA plasmid; 250 µCi 35S-UTP (1100 to 1300 Ci/mmol); 500 µM each of ATP, CTP, and GTP; 40 µM RNASin; 10 mmol/L DTT; 40 mmol/L Tris and 10 µl of either T7 polymerase (for sense transcriptions) or SP6 polymerase (for antisense transcriptions). After a 60- to 75-minute incubation at 37°C, the DNA template was digested by addition of 1 U DNase (Promega) and incubation at 37°C for an additional 15 minutes. Free nucleotides were then separated on a Sephadex G-50 column. Specific activity of the probes ranged from 5 to 15 x 10^7 cpm/mg. Probes were stored at -20°C and used within 24 hours of synthesis.

In Situ Hybridization

Myocardial tissue that had been fixed in 10% NBF and embedded in paraffin was deparaffinized following standard protocol. The tissue sections were washed with 0.5 x SSC (1 x SSC is 150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7.0), digested with proteinase K (1 µg/ml; Sigma) in Rnase A (Promega) buffer, and then postfixed for 10 minutes in paraformaldehyde. Several 0.5 x SSC washes were followed by prehybridization for 2 hours in 50 µl prehybridization buffer (0.3 mol/L NaCl; 20 mmol/L Tris, pH 8.0; 5 mmol/L EDTA; 1 x Denhardt's solution; 10% dextran sulfate; and 10 mmol/L DTT). The hybridizations were started by adding 5 x 10^6 cpm of 35S-labeled riboprobe in 50 µL of prehybridization buffer and allowed to proceed overnight at 50°C. After hybridization, the sections were washed with 0.5 x SSC, treated with Rnase A (20 µg/ml for 30 minutes at room temperature), and washed in 2 x SSC (twice for 2 minutes each) followed by several 2 x SSC washes. After the tissue was air dried, it was dipped in NTB2 nuclear emulsion (Kodak) and exposed in the dark at 4°C for 2 to 4 weeks. After the sections were developed they were counterstained with either hematoxylin/eosin or methyl green, dehydrated, and mounted.

Quantitative In Situ Hybridization

Quantitative in situ hybridization was carried out by performing hybridizations with antisense and sense (control) riboprobes on adjacent myocardial sections from each patient. Radioactivity was determined (after a 4.5-hour exposure of the sections) on a model 400A PhosphoImager (Molecular Dynamics) maintained by the Markey Molecular Medicine Facility at the University of Washington. Compared with radiographic film, phosphorus plate imaging has significant advantages because detection of the radioisotope is linear over time rather than sigmoidal and the sensitivity for detection of 32P is 100-fold greater. After exposure on the PhosphoImager, total counts were obtained for sample volumes from adjacent tissue sections hybridized with either the antisense or sense (control) riboprobes. To determine background radioactivity, total counts were also obtained for an equivalent sample volume placed next to the tissue sections. The values for background radioactivity were subtracted from the values for sense and antisense hybridizations. Specific hybridization was determined by subtracting values obtained for the sense riboprobe from values for the antisense riboprobe, and the results were expressed as counts per pixel. The coefficient of variation for quadruplicate determinations of specific LPL signal on adjacent tissue section pairs from 10 separate tissue samples was 23.1%.

Statistical Analyses

To test for significant differences in LPL mRNA expression (as determined by in situ hybridization) among the 25 patients with cardiomyopathy, the nonparametric Mann-Whitney U test was used for the following variables: (1) NYHA functional class III versus class IV congestive heart failure and (2) current therapy with or without intravenous β-adrenergic agonists (dopamine and/or dobutamine). To determine whether LPL expression was related to the degree of left ventricular dysfunction, plots of ranked LPL values versus left ventricular ejection fraction were compared, and nonparametric Spearman correlation coefficients were determined for (1) all 25 cardiomyopathy patients, (2) all subjects by functional class, and (3) all subjects by β-adrenergic agonist use. Statistical analyses were performed with SPSS/PC+ software. Statistical significance was set at P < .05.

Results

Immunohistochemistry

LPL protein, as detected immunohistochemically with rabbit polyclonal antiserum LPL-5780 (Fig 1a) or
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with mouse monoclonal antibody 5F9 (Fig 1b and 1c), was present in association with cardiac myocytes, endothelial cells, adipocytes, and interstitial cells identified histologically as vascular pericytes and medial smooth muscle cells (Fig 1a and 1b) in both cardiomyopathy patients (Fig 1a and 1b) and control patients (Fig 1c). Specificity of the polyclonal LPL antiserum and of the monoclonal antibody was confirmed by comparison with adjacent sections stained with either normal rabbit serum or normal mouse serum, as appropriate.

In Situ Hybridization of Human Myocardium

To determine the cellular source of LPL in humans, in situ hybridization was performed on myocardial segments from patients with cardiomyopathy (Table). Myocardium from these cardiomyopathy patients was used primarily because all samples had been fixed in 10% NBF within 2 hours of cardiac asystole, thus minimizing both the degree and variability of both mRNA and protein degradation. Thus, it was thought that this quickly preserved myocardium would have the greatest likelihood of having detectable amounts of LPL mRNA in all cell types that might express it. With use of an antisense riboprobe, LPL mRNA was detected only in cardiac myocytes and not in interstitial cells (Fig 2a). A negative-control hybridization performed with a sense riboprobe on an adjacent section showed no specific hybridization (Fig 2b).

Short-term treatment with agents that stimulate adenylate cyclase via β-adrenergic receptor stimulation, before removal of their hearts. However, LPL mRNA was detectable only in association with cardiac myocytes and not with interstitial cells in these six patients (Fig 2c). Finally, LPL mRNA was also detected in epicardial adipocytes (Fig 2d).

To exclude congestive heart failure as an explanation for the lack of LPL expression by interstitial cells, myocardial samples were examined from seven control patients who were donors of organs other than the heart. In contrast to the cardiomyopathic myocardial specimens, which all had ischemic times of ≤32 hours, ischemic times for control patients varied from 9 to 38.5 hours. Nonetheless, LPL mRNA was detectable only in cardiac myocytes by in situ hybridization in all seven cases (data not shown).

Quantitative In Situ Hybridization

By the nonparametric Mann-Whitney U test, there were no significant differences in myocardial LPL mRNA expression between NYHA functional class III versus class IV (P = .75) and patients receiving intravenous β-adrenergic agonists versus those who were not (P = .51). Likewise, the nonparametric Spearman correlation coefficients showed no significant relationships between plots of ranked LPL values versus left ventricular ejection fraction neither for all 25 subjects (r = .20) nor for all subjects with respect to NYHA functional class or β-adrenergic agonist use.

Comparison of Myocardial LPL mRNA Expression for Patients With or Without Cardiomyopathy

Ischemic times for myocardial specimens (defined as the time between onset of cardiac asystole and place-
### Characteristics of Cardiomyopathy Patients

<table>
<thead>
<tr>
<th>Recipient Heart No.</th>
<th>Type</th>
<th>Gender, M/F</th>
<th>NYHA Functional Class</th>
<th>β-Adrenergic Agonist Use</th>
<th>LVEF</th>
<th>LPL mRNA, Counts/ Pixel</th>
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<td>CM-1</td>
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<td>No</td>
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<td>29.78</td>
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NYHA indicates New York Heart Association; LVEF, left ventricular ejection fraction; LPL, lipoprotein lipase; I, ischemic; and N, nonischemic.

Discussion

The present study demonstrates that LPL mRNA, as determined by in situ hybridization, is produced by cardiac myocytes in humans. This observation is consistent with the results of in situ hybridization in guinea pigs, which have shown that LPL mRNA is present in association with cardiac myocytes, although others have used the same technique to demonstrate that LPL mRNA is produced primarily by interstitial cells in rats. However, the authors of the latter study, as well as others, have shown that isolated rat cardiac myocytes do produce LPL in culture. Thus, in vitro studies suggest that cardiac myocytes of rats should produce LPL, as has been demonstrated previously for guinea pigs. The present study demonstrates that LPL mRNA detectable by in situ hybridization was apparently linear ($r = .73, P < .001$), with a half-life of approximately 26 hours. LPL mRNA values for each of seven control patients were plotted against their respective ischemic times (open circles). When plotted as a function of ischemic time, there was no apparent difference between the values for LPL mRNA from these three cardiomyopathic patients and control patients.

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pig cardiac myocytes, and as is demonstrated in the present study for human cardiac myocytes.

In the present study, LPL mRNA was not detected by in situ hybridization in association with interstitial cells, as has been reported previously in rats but not guinea pigs. In rats, treatment with cholera toxin, which stimulates adenylate cyclase, increases myocardial LPL mRNA production, as determined by Northern blot analysis, and increases the in situ hybridization signal for LPL mRNA in interstitial cells. Six of the cardiomyopathy patients in the present study had been receiving chronic continuous intravenous treatment with β-adrenergic agonists, which stimulate adenylate cyclase, albeit through a different mechanism than does

Fig 2. Hybridization of an antisense lipoprotein lipase (LPL) riboprobe on human myocardium from a patient with cardiomyopathy (a) demonstrates the presence of LPL mRNA (black silver grains) in association with cardiac myocytes but not with other cell types. Negative-control hybridization with a sense LPL riboprobe on an adjacent section from the same patient (b) shows no specific hybridization. In a cardiomyopathy patient receiving high-dose (13 µg·kg⁻¹·min⁻¹ IV) therapy with the β-adrenergic agonist, dobutamine, the antisense LPL riboprobe detects LPL mRNA only in association with cardiac myocytes but not with interstitial cells (c), despite treatment with this adenylate cyclase-activating agent. Specific hybridization of the LPL riboprobe was also seen in association with adipocytes (A) in addition to myocytes (M) (d). In normal control myocardium, LPL mRNA was also detected only in association with cardiac myocytes (not shown). Interstitial cells and arterioles have no specific hybridization for LPL mRNA (not shown). [Hematoxylin/eosin counterstain; original magnifications ×400 (a, b), ×200 (c), and ×630 (d).]

Fig 3. Hybridizations were performed on sections of myocardium (obtained from one patient) placed in neutral-buffered formalin at different times after onset of cardiac asystole (different ischemic times). Much more lipoprotein lipase (LPL) mRNA was detected in the sample with a short ischemic time of 1.5 hours (a) compared with a sample with a longer ischemic time of 12 hours (b), demonstrating that mRNA detectable by in situ hybridization decreases as ischemic time increases. (Hematoxylin/eosin counterstain, original magnification ×100.)
The amount of lipoprotein lipase (LPL) mRNA detectable by in situ hybridization for three cardiomyopathy patients (CM-23, CM-24, and CM-25) is plotted as a function of ischemic time (o, , ). The amount of detectable LPL mRNA decreases in an apparently linear fashion (- - - - indicates the regression line; solid line, the 95% confidence intervals for the regression) as ischemic time increases (r = .73, P < .001) with a disappearance half-time of ~26 hours. Values for LPL mRNA detectable by in situ hybridization from seven different control patients are also plotted as a function of ischemic time (o), and comparison of these values with the regression line (- - - -) for the values of the three cardiomyopathy patients reveals no apparent difference in detectable levels of myocardial LPL mRNA between these three cardiomyopathy patients and control patients.

cholera toxin. However, LPL mRNA was not detected in interstitial cells from any of these patients. It is possible that short-term adenylate cyclase stimulation increases LPL mRNA expression by interstitial cells, while long-term stimulation does not. However, LPL mRNA was also not detected in interstitial cells of myocardium from seven patients without overt congestive heart failure and whose circulating levels of catecholamines were likely to have been high as a result of recent traumatic injury. These findings do not exclude the possibility that interstitial cells might have been expressing LPL mRNA at levels below the threshold of detection by our riboprobe. Regardless, on the basis of the results of this study, we conclude that LPL mRNA is produced primarily by cardiac myocytes in human myocardium.

Statistical analyses comparing baseline clinical characteristics, including NYHA functional class, β-adrenergic agonist treatment, and left ventricular ejection fraction, with the amount of mRNA detected in each sample of myocardium from the 25 patients with cardiomyopathy revealed no statistically significant relationships between any of these clinical variables and the amount of expressed LPL mRNA. However, there are several reasons why these results should be interpreted with caution. First, our technique necessitated that the mRNA detected be expressed as a function of area, without normalization for cardiac myocyte cell number or mass. Because cardiomyopathy is associated with increases in fibrosis and with myocyte cell loss and hypertrophy, it was not possible with these methods to determine whether the amount of LPL mRNA expressed per cell differs in patients with cardiomyopathy compared with control patients. However, the data do represent the total amount of mRNA available for production of LPL per area of myocardium. Second, there was wide variation in the absolute values of LPL mRNA detected, possibly due to differences in mRNA preservation among specimens and/or variability in technique, in addition to potential interindividual biological variation in LPL mRNA expression.

The wide variability in mRNA expression observed among individuals suggests that LPL may not be an essential component of myocardial metabolism. This conclusion is also supported by the observation that although many LPL-deficient individuals have been identified, there are no reports that have associated LPL deficiency with overt abnormalities in myocardial function. However, the possibility still exists that LPL expression represents a compensatory mechanism by which failing or energy-starved hearts could increase the amount of substrate available for oxidative metabolism.

The reasons why cardiac myocytes express LPL are not known. In addition to the possibility that cardiac myocytes use LPL to liberate fatty acids for oxidation, it may also be that LPL is used by cardiac myocytes as a ligand for non-receptor-mediated uptake of lipoproteins. Studies in other cell types have demonstrated that LPL can mediate uptake of a number of plasma lipoproteins, including VLDL, low-density lipoprotein (LDL), and lipoprotein(a), via binding to heparan sulfate moieties on the cell surface. Also, a recent study has demonstrated that LPL may mediate translocation of cholesterol ester from β-VLDL into smooth muscle cells and fibroblasts without receptor-mediated endocytosis. LPL-mediated receptor-independent uptake of lipoproteins or cholesterol esters could provide a mechanism by which cardiac myocytes increase the amount of lipoprotein lipid available for metabolism or membrane synthesis.

In summary, the present study documents that LPL is synthesized primarily by cardiac myocytes in human myocardium in patients with and without overt cardiomyopathy. In addition, LPL mRNA was not detected in cardiac interstitial cells, even in patients treated with β-adrenergic agonists. No obvious association was found between the amount of LPL mRNA detected by in situ hybridization and several clinical variables related to the severity of congestive heart failure. In addition, the time course of degradation of LPL mRNA detectable by in situ hybridization was determined, and with this information, no obvious differences in myocardial LPL mRNA expression were found between patients with cardiomyopathy and control patients. Finally, immunohistochemistry with LPL-specific antibodies localized LPL protein to endothelial cells and adipocytes, as well as to interstitial cells and cardiac myocytes.

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