Expression of Lipoprotein Lipase mRNA in Rat Heart Is Localized Mainly to Mesenchymal Cells as Studied by In Situ Hybridization

Olga Stein, Yechezkiel Stein, Sigal Pressman Schwartz, Ayeleth Reshef, Tova Chajek-Shaul, Mazal Ben-Naim, Gideon Friedman, and Eran Leitersdorf

The expression of lipoprotein lipase mRNA (LPL mRNA) was studied in rat hearts by use of a sulfur-35-labeled antisense mRNA probe. Rats were studied under three conditions: fed, fasted, and injected with cholera toxin (an irreversible agonist of adenylate cyclase) and then fasted. The highest LPL activity was found in the hearts of cholera toxin-injected, fasted rats. After injection of cholera toxin, LPL mRNA levels were 3.5-fold higher than those from fed rats. Using in situ hybridization, we studied the site of expression of LPL mRNA under the same three experimental conditions. In sections of hearts from cholera toxin-injected, fasted rats, concentrations of autoradiographic grains, representing the site of LPL mRNA, were seen over interstitial elements, which comprise capillary and perivascular cells. A more diffuse and sparse reaction was seen over cardiac myocytes and was not always distinguishable from background. A similar but much less definitive localization was seen in sections of hearts from fasted rats. The present results indicate that in the rat heart, the main site of LPL synthesis and processing, especially after stimulation with an irreversible agonist of adenylate cyclase, is localized to interstitial elements rather than to adult cardiac myocytes. (Arteriosclerosis and Thrombosis 1991;11:857-863)

Lipoprotein lipase (LPL), the key enzyme in the metabolism of plasma lipoproteins, interacts with its substrate on the surface of vascular endothelium. We have shown previously that in the rat heart, LPL activity is low in the fetus and that it increases linearly between day 1 and day 20 after birth. More recently, this finding was extended to LPL mRNA, indicating that the rise in LPL activity with age is under transcriptional control. During postnatal development, morphological changes are seen in the rat heart, of which the increase in the capillary bed is a prominent one, and we have related the rise in heart LPL activity to this morphological change. The concomitant increase in LPL mRNA level under those circumstances suggested to us that the main site of LPL synthesis may not be the adult cardiac myocyte but rather the interstitial cells of the capillary bed such as pericytes. In the present study, we have attempted to localize the site of LPL mRNA expression in the rat heart by use of in situ hybridization. In our previous studies the highest LPL activity in the heart in vivo could be induced by injection of cholera toxin, an irreversible agonist of adenylate cyclase. Because the increase in LPL activity was not explained by a slight prolongation of the 1/2 of LPL activity, we postulated that cholera toxin induced an increase in LPL synthesis, and hence we have used this model system in our present approach.

Methods

Animals

Male albino rats of the Hebrew University Sabra strain, weighing 100–120 g, were used. Female mice of the C-57 black strain were also used when they were 2–3 months of age. The animals were kept in individual cages in light- and temperature-controlled rooms. The rats were examined concurrently under three experimental conditions: groups of two to three
rats were either fed ad libitum, fasted for 18 hours, or injected intravenously with cholera toxin (50 μg/100 g body wt) and then fasted for 18 hours. The experiment was repeated three times. While the animals were under ether anesthesia, they were exsanguinated from the aorta, the tissues were removed, and portions were taken for RNA extraction, determination of LPL activity, and in situ hybridization.

**Probe Preparation**

Hybridization probes were prepared by use of plasmid pBSLPL, which includes a 213-bp fragment of genomic sequences from exon 5 of the mouse LPL gene. This fragment was isolated from a 1.3-kb Xba I fragment of LPL cDNA and subcloned into pBS vector (Stratagene, La Jolla, Calif.). Sequence analysis confirmed that the clones were identical to those published. Sense and antisense probes were prepared by linearizing the constructs with an appropriate restriction endonuclease and synthesizing sulfur-35 uridine triphosphate–labeled RNA transcripts (Stratagene RNA transcription kit). After probe preparation, the labeled transcripts were separated by gel electrophoresis, blotted onto a nylon membrane (Biotrans nylon membrane, ICN Biochemical, Irvine, Calif.), and autoradiographed. Only probes that consisted of a single band with the expected size on autoradiography were used for the RNA and in situ experiments. β-Actin probe was prepared by primer extension.

**RNA Blotting Analysis**

Total RNA was extracted from rat heart, liver, and fat tissue in 4 M guanidine thiocyanate as described. The RNA was denatured in 2.3 M glyoxal and subjected to electrophoresis on 1.6% agarose gel as described by McMaster and Carmichael. The RNA was transferred to a nylon-based membrane (Biodytron nylon membrane, ICN Biochemical, Irvine, Calif.), and autoradiographed. Only probes that consisted of a single band with the expected size on autoradiography were used for the RNA and in situ experiments. β-Actin probe was prepared by primer extension.

**Slot Blots**

RNA in 50% formaldehyde and 10×SSC was applied to a nylon membrane by use of a slot-blot apparatus (Schleicher and Schuell, Keene, N.H.). The membranes were probed as described above.

**Preparation of Tissue for In Situ Hybridization**

The heart, liver, and lung were washed in ice-cold phosphate-buffered saline (PBS) and fixed in freshly prepared 4% paraformaldehyde in PBS for 24 hours at 4°C. After dehydration in graded ethanol, the tissue was embedded in paraffin, and the blocks were kept at 4°C. Sections about 10 μm thick were collected on precleaned gelatin “subbed” slides and were stored at 4°C until hybridization. The sections were pretreated for hybridization according to Klein et al. After deparaffinization the slides were fixed in 4% paraformaldehyde, permeabilized with proteinase K, treated with acetic anhydride, and dehydrated. The probe was prepared for in situ hybridization at 1×10^6 cpm/slide in 10 mM dithiothreitol, 50% formamide, 0.3 M NaCl, 20 mM tris(hydroxymethyl)aminoethane (Tris), 5 mM EDTA, 10 mM phosphate buffer, 1×Denhardt’s solution, 0.5 mg/ml yeast RNA, and 10% dextran sulfate and was warmed to 80°C. Hybridization was performed under stringent conditions (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1×Denhardt’s solution, 0.5 mg/ml yeast RNA, and 10 mM dithiothreitol at 52°C) for 18 hours. Thereafter, the slides were washed in 2×SSC, 25 mM dithiothreitol, and 50% formamide at 65°C for 30 minutes, washed again with 10 mM Tris (pH 7.5), 0.005 M EDTA, and 0.4 M NaCl, and treated with ribonuclease A for 40 minutes. After two washes with 2×SSC and 0.1×SSC for 15 minutes at 37°C, the slides were dehydrated in graded ethanol containing 0.3 M ammonium acetate. Autoradiography was performed with Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, N.Y.); the slides were exposed for up to 4 days at 4°C, developed in Kodak D-19 developer at 18°C, fixed with Kodak fixer, washed, stained with hematoyxin and eosin, and mounted in Permount (Fisher Scientific, Fairlawn, N.J.).

**Cell Cultures and Lipoprotein Lipase Determination**

Mouse peritoneal macrophages were prepared as described previously. Peritoneal exudate macrophages were harvested from mice 4 days after intraperitoneal injection of 1 ml thioglycolate. Cells were washed with Ca²⁺,Mg²⁺-free PBS, resuspended in Dulbecco’s modified minimum essential medium containing 10% fetal bovine serum, and plated at a density of 2×10⁶ cells/dish in 35-mm Falcon (Becton Dickinson, Cowley, Oxford, England) tissue-culture dishes; nonadherent cells were removed after incubation for 2 hours at 37°C. F₁ heart cell cultures, consisting mainly of mesenchymal cells, were prepared from 2-day-old rat hearts as described previously and were cultured for 1 week in Dulbecco’s minimal essential medium containing 10% horse serum and 10% fetal bovine serum.

LPL was determined on homogenates of rat heart, cultured cells, and media. Twenty- to fifty-milligram aliquots of heart were homogenized in 4 ml 0.025 M NH₄/H₄Cl buffer (pH 8.1) by use of the Polyrtron homogenizer (Kinematica, Lucerne, Switzerland) with a pt10-11 probe at maximum speed for 2 minutes at 0°C. Cells were released from the petri dish with a rubber policeman and homogenized with an all-glass Kontes (Kontes, Vineland, N.J.) conical homogenizer in 1 ml of the same buffer. Determination of LPL activity was performed with 0.1 ml homogenate or 0.1 ml medium, and 0.1 ml substrate containing tritium-
Expression of LPL mRNA in Rat Heart

FIGURE 1. Agarose gel electrophoretograms showing expression of lipoprotein lipase mRNA in rat tissues. RNA was prepared from fasted, fed, or cholera toxin-treated, fasted rats. Total RNA was prepared from heart, adipose tissue, and liver (see “Methods”). Total glyoxalated RNA (15 μg) was subjected to electrophoresis on agarose gels and blotted onto nylon membranes. The membrane was probed with 1×10⁶ cpm/ml probe and autoradiographed for 24 hours at room temperature. Band size was calculated according to an 18S and a 28S ribosomal RNA marker. RNA quantification was verified by reprobing with β-actin. FE, fed; FA, fasted; CT, cholera toxin treated.

labeled triolein prepared according to the method of Nilsson-Ehle and Schotz. The specific radioactivity of the triolein moiety was 300–350 dpm/nmol triacylglycerol. Incubations were performed at 37°C for 60 minutes. The reaction was stopped by addition of CH₃OH/CHCl₃/heptane (1.4:1.25:1, vol/vol/vol), and the extraction of fatty acids and calculation of enzyme activity were performed according to Nilsson-Ehle and Schotz.

Materials

Cholera toxin (C-3012), triolein, and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) were obtained from Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases and other enzymes were obtained from New England Biolabs, Beverly, Mass., and from Boehringer Mannheim, Mannheim, FRG. [³²S]Juridine triphosphate was purchased from New England Nuclear, Boston, Mass. [³H]triolein was purchased from Amersham International, Amersham, UK. Biotrans nylon membranes were purchased from ICN.

Results

Lipoprotein Lipase mRNA Levels and Lipoprotein Lipase Activity

The expression of LPL mRNA was studied in the heart, adipose tissue, and liver from rats that were fed ad libitum, fasted for 18 hours, or injected with cholera toxin and then fasted for 18 hours. In the RNA blot analysis (Figure 1), the 213-bp LPL probe mainly identified a 3.6-kb mRNA and a faint 1.7-kb band. Expression of LPL mRNA was not observed in liver RNA samples. Relative quantification of LPL mRNA in the heart under the three experimental conditions was performed by slot-blot analysis. As shown in Figure 2, fasting and injection of cholera toxin resulted in 2.4-fold (11.6 units/μg versus 4.8 units/μg) and 3.5-fold (16.7 units/μg versus 4.8 units/μg) increases in heart LPL mRNA, respectively, when compared with hearts from fed rats. Densitometry of the autoradiograph of the same mem-

TABLE 1. Effect of Cholera Toxin on Lipoprotein Lipase Activity in Rat Heart and Serum

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Experiment No.</th>
<th>Heart (milliunits/g)</th>
<th>Serum (milliunits/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>1</td>
<td>733</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>634</td>
<td>0.8</td>
</tr>
<tr>
<td>Fasted</td>
<td>1</td>
<td>1,350</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,200</td>
<td>2.3</td>
</tr>
<tr>
<td>Fasted+cholera toxin</td>
<td>1</td>
<td>2,466</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2,333</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Rats were fed ad libitum, fasted for 18 hours, or injected with cholera toxin (50 μg/100 g body wt) and then fasted for 18 hours. Values are means of duplicate determinations that varied less than 10%. 1 milliunit=1 nmol fatty acid released/min.
LPL activity was determined in rat hearts under the same three experimental conditions, and results of two experiments are shown in Table 1. In both instances, the enzyme activity was as much as fourfold higher in the cholera toxin–treated, fasted rat hearts when compared with that of the fed state. A similar increase was also seen in serum LPL activity.

brane hybridized with a β-actin probe showed a slightly lower signal in hearts from the cholera toxin–treated and fasted rats compared with hearts from the fed rats (1.4 units/μg and 1.8 units/μg, respectively). In another experiment, a twofold increase occurred in the fasted rat heart LPL mRNA when compared with that of the fed state.

Figures 3–6. Autoradiographs of sections of cholera toxin–treated, fasted rat hearts hybridized with sulfur-35–labeled antisense lipoprotein lipase probes. Sections are representative of findings obtained in several hybridizations and show a diffuse reaction over and between myocytes. Arrows indicate foci of concentration of autoradiographic grains, which are localized to interstitial elements that could be perivascular or vascular cells. ×384.
In Situ Hybridization

Because the highest amount of LPL mRNA and LPL activity was observed in hearts from cholera toxin–treated, fasted rats, we focused mainly on these hearts for in situ hybridization. Hearts from six cholera toxin–treated, fasted rats, three fasted rats, and three fed rats were examined; each heart was divided into four blocks, and approximately 150 sections were hybridized in six different hybridization experiments. In each experiment, serial sections were hybridized to a sense RNA probe that served as a control. In some experiments, we also examined sections of liver and lung. The results obtained in sections of hearts from the cholera toxin–treated, fasted rats are shown in Figures 3–6. In all the sections examined by in situ hybridization, we observed foci of autoradiographic grains that were localized over interstitial cells, which could be either vascular or perivascular rather than cardiac myocytes. The reaction over the myocytes was diffuse and difficult to distinguish from background label seen between the myocytes. In sections from fasted rat hearts, similar foci of grains, although less prominent, could be discerned in some sections, whereas the reaction over the myocytes was difficult to distinguish from the background label (Figure 7). The same was also true for sections of hearts from fed rats. Sections hybridized with the sense probe usually gave low background signals (Figure 8).

Lipoprotein Lipase Activity in Cultured Cells

Because the cells in the heart interstitium may have been macrophages, which are also known to synthesize LPL,9 we compared the response of F1 mesenchymal heart cell cultures and macrophages to dibutyryl cyclic AMP12,13 and HEPES.14 In accordance with our previous findings, in the mesenchymal heart cells there was a significant increase in LPL activity after both treatments,12,13 while a reduction of enzyme activity in dibutyryl cyclic AMP–treated cells was observed in peritoneal macrophages (Table 2). Therefore, these results do not support the possibility that the foci of autoradiographic grains found in the sections of heart were over macrophages.

**TABLE 2. Effect of Dibutyryl Cyclic AMP and HEPES on Lipoprotein Lipase Activity in Cultured F1 Heart Cells and Peritoneal Macrophages**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>F1 cultures</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Db cAMP</td>
<td>171±9</td>
<td>9±0.3</td>
</tr>
<tr>
<td>HEPES</td>
<td>311±29</td>
<td>68±1.0</td>
</tr>
</tbody>
</table>

F1 heart cell cultures were used 1 week after plating, and peritoneal macrophages were used 24 hours after plating. Cultures were exposed to 0.45 mM dibutyryl cyclic AMP (Db cAMP) for 24 hours or to 100 mM HEPES (pH 7.4) for 48 hours. Values are mean±SEM of triplicate dishes compared with their respective controls, which were taken as 100%.

LPL, lipoprotein lipase; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

Discussion

Localization of LPL mRNA by use of in situ hybridization techniques was reported in selected rat tissues such as the brain cortex, the intermediate lobe of the pituitary gland, and special areas of the adrenal and kidney medullas.15 However, even though the authors examined other tissues in which LPL mRNA has been reported (presumably also the rat heart), they stated that “because of high amounts of background definitive localization was not demonstrated.”15

In the present study, we have observed that injection of cholera toxin to rats results in an increase in heart LPL activity and LPL mRNA levels. This increase permitted us to localize the site of LPL mRNA expression to interstitial cellular elements in preference to cardiac myocytes. The interstitial cellular elements in the myocardium comprise fibroblasts, pericytes, and capillary endothelial cells. These cells account for about 6% while myocytes account for 69% of the total volume of the myocardium, as determined by stereological quantification in the rabbit heart.16 The predominant localization of LPL mRNA described presently supports our contention that LPL synthesis in cultured cells derived from 2-day-old rat hearts occurs mainly in mesenchymal cells from the interstitium rather than in beating cardiac myocytes.10 A similar conclusion has also been drawn with respect to basic fibroblast growth factor.17 Use of the preplating technique has shown that basic fibroblast growth factor mRNA was expressed in the preplates (nonmyocytes) and appeared in myocyte cultures only after some days of cultivation due to proliferation of nonbeating cells. On the other hand, acid fibroblast growth factor mRNA was expressed in cardiac myocytes from the beginning of the culture.17 However, a recent study has shown that in skeletal muscle both acidic and basic fibroblast growth factor mRNA are expressed by proliferating satellite cells but not by adult myotubes.18 It needs to be emphasized that the presently reported localization of LPL mRNA is at variance with a recent report by Camps et al.19 In that study it was concluded that “hybridization for LPL mRNA was over muscle cells” of nonfasted guinea pig hearts. This apparent controversy could have been due to the difference in species as well as in our experimental conditions, that is, treatment with cholera toxin, but perhaps also to a difference in the interpretation of results. Our data do not exclude the expression of LPL mRNA in myocytes but do provide evidence that under conditions of stimulation, interstitial cells respond with an increased expression of LPL mRNA. In this context it seems pertinent to refer to a recent study by Carroll et al20 of LPL activity in isolated adult cardiac myocytes. In these cells LPL activity did not respond to cholera toxin.20 These results further support our present findings that the increase in LPL mRNA after cholera toxin injection occurred in interstitial cells and not in cardiac myocytes. In view of the well-established
FIGURE 7. Autoradiograph of section of fasted rat heart hybridized with sulfur-35-labeled antisense lipoprotein lipase probe. Section is representative of findings obtained in several hybridizations and shows a diffuse reaction over and between myocytes. Arrows indicate foci of concentration of autoradiographic grains localized to interstitial elements that could be perivascular or vascular cells. x384.

Evidence that LPL mRNA has a signal sequence and that the enzyme requires a secretory pathway for its release, it appears that cellular elements that do have a well-developed rough endoplasmic reticulum and Golgi apparatus would be more suitable candidates for the synthesis and processing of LPL than would adult myocytes; the latter produce predominantly contractile proteins, such as actin and myosin, or cell surface receptors. There is, however, an example of a secretory product that is synthesized in the myocyte, that is, the atrial natriuretic polypeptide. These cells that express atrial natriuretic polypeptide mRNA and synthesize atrial natriuretic peptide have a well-developed rough endoplasmic reticulum, a voluminous Golgi apparatus, and prominent secretory granules. Immunolocalization of LPL in mouse heart has been performed by Blanchette-Mackie et al, and these authors have concluded that LPL is synthesized by cardiac myocytes. However, their findings render themselves amenable to another interpretation, namely, endocytosis of surface-bound LPL rather than secretion of LPL. The presence of gold particles in various vesicles and in the Golgi apparatus is reminiscent of the findings reported for transferrin receptors that were tagged at the surface with antitransferrin receptor antibody; the radiolabel was subsequently detected in all Golgi elements.

FIGURE 8. Autoradiograph of section of cholera toxin-treated, fasted rat heart hybridized with sulfur-35-labeled sense lipoprotein lipase probe. Sections (including this one) hybridized with the sense probe gave low background. x384.

The reciprocal regulation of heart and adipose tissue LPL is a well-documented phenomenon, but the mechanism of such regulation has not yet been agreed on. Thus, in adipose tissue of guinea pigs fasted for 48 hours, LPL activity, LPL synthetic rate, and LPL mRNA were lower than after feeding. Similar findings were also reported in chicken adipose tissue. However, in adipose tissue of rats fasted for 12 hours, both the rate of LPL synthesis and LPL mRNA levels were higher, while the enzyme activity was lower than after a 24-hour fast and refeeding for 12 hours. The authors concluded that the regulation of LPL was controlled posttranslationally. This complexity appears to be even greater in the heart. Whereas in the guinea pig there was no difference in either LPL activity or LPL mRNA levels between the fed and fasted state, in the chicken heart the LPL mRNA level was much lower in the fed than in the fasted state, suggesting pretranslational regulation. In the rat heart, however, even though high enzyme activity and LPL protein were seen in the fasted compared with the fed state, the fasted to fed ratio of LPL mRNA was 0.95. In the present study, we have used a different experimental design and compared LPL activity and LPL mRNA levels in animals either fasted overnight or fed ad libitum. In fasted rats or in rats injected with cholera toxin and then fasted, heart LPL activity and LPL mRNA level were significantly higher than in the fed state.

Because LPL in the vascular compartment is found not only on the endothelial cell surface but also in...
plasma, it seems of interest that the rise in plasma LPL parallels a rise in heart LPL but not in adipose tissue LPL. One of the mechanisms involved in the release of LPL into the circulation could be through the action of a phosphatidylinositol-specific phospholipase C or D, which is known to release heparan sulfate proteoglycan from its phosphatidylinositol anchor. The LPL being bound to the heparan sulfate from the surface serves as a signal for new enzyme synthesis, possibly by derepression of LPL mRNA synthesis. We have encountered such a situation in cultures of F1 heart cells, in which addition of 100 mM HEPES to the culture medium resulted in a very significant release of LPL, which was followed by an increase in enzyme synthesis as well as a rise in LPL mRNA level (O. Stein, preliminary observations).

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