Fatty acids reduce heparin-releasable LPL activity in cultured cardiomyocytes from rat heart

LORRAINE G. ANDERSON, ROGAYAH CARROLL, H. STEPHEN EWART, ANJLI Acharya, and DAVID L. SEVERSON
Smooth Muscle Research Group, Faculty of Medicine, The University of Calgary, Calgary, Alberta, Canada T2N 4N1

Fatty acids reduce heparin-releasable LPL activity in cultured cardiomyocytes from rat heart. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E759–E767, 1997.—Varying glucose and fatty acid (FA) concentrations in the medium of cultured cardiomyocytes from adult rat hearts were tested for effects on lipoprotein lipase (LPL) activity. Glucose (5.5, 11, and 25 mM in the culture medium for 18–22 h) had no effect on either heparin-releasable LPL (HR-LPL) or on cellular LPL (C-LPL) activities. When cardiomyocytes were cultured overnight with 60 µM oleate, HR-LPL activity was reduced to 20% of control, with no change in C-LPL activity or total C-LPL mass. Similar results (HR-LPL and C-LPL activities) were obtained with 60 µM concentrations of palmitate and myristate; linoleate and eicosapentaenoate did reduce C-LPL activity, but the decrease in HR-LPL activity was much greater. Oxfenicine, an FA oxidation inhibitor, did not alter the inhibitory effect of 60 µM oleate on HR-LPL. Short-term incubations (1 and 3 h) of cultured cardiomyocytes with 60 µM oleate did not displace LPL into the medium. Immunodetectable LPL on the cell surface of oleate-treated cultured cardiomyocytes was increased compared with control cells, but heparin treatment released the same amount of LPL mass that had reduced catalytic activity.

myocardial cells; immunohistochemistry

IN THE ADULT HEART lipoprotein lipase (LPL) is synthesized and processed in cardiomyocytes and then is translocated to binding sites on the luminal surface of endothelial cells in the coronary vasculature (4, 6, 8, 20). The functional endothelium-bound enzyme catalyzes the hydrolysis of the triacylglycerol (TG) component of circulating lipoproteins (21); nonesterified fatty acids (FA) produced by the action of LPL are then available for oxidation in cardiomyocytes.

Hypertriglyceridemia is a characteristic feature of insulin-dependent diabetes mellitus, principally because of reduced catabolism of TG-rich lipoproteins (28). The degradation of TG-rich lipoproteins was decreased in diabetic perfused hearts (22, 23) as a consequence of reduced functional endothelium-bound LPL activity (7, 17, 23). In addition, LPL activity in cardiomyocytes, the precursor of the functional enzyme on the vascular endothelium, was decreased after the acute induction of insulin-deficient diabetes (5, 7, 9, 17). Administration of insulin in vivo to diabetic rats rapidly reversed the diabetes-induced decrease in LPL activity in cardiomyocytes, but in vitro incubations of cardiomyocytes from either control or diabetic rat hearts with insulin had no direct stimulatory effect on LPL activity (5, 7), despite the presence of functional insulin receptors in the cardiomyocyte preparations. Therefore, the inhibitory effect of diabetes on LPL activity in isolated cardiomyocytes may not be a direct consequence of insulin deficiency but rather secondary to some other metabolic factor(s) that is altered in an acute, streptozotocin-induced model of diabetes. Hyperglycemia and increased circulating FA concentrations as a result of unrestrained adipose tissue lipolysis are common metabolic features of insulin-deficient diabetes. Several investigations have reported that LPL activity in cultured adipocytes was decreased when the glucose concentration was increased (15) or when FA were added to the culture medium (2, 16). Therefore, the objective of the present study was to determine the effect of glucose and FA on LPL activity in cultured cardiomyocytes from rat heart. Heparin can displace the portion of total cellular LPL activity that is bound to heparan sulfate proteoglycan (HSPG) binding sites on the surface of cells (6). This heparin-releasable LPL (HR-LPL) activity was selectively reduced when cardiomyocytes were cultured in the presence of FA.

MATERIALS AND METHODS

Isolation, culture, and incubation of cardiomyocytes from rat hearts. Adult rat ventricular cardiomyocytes were isolated essentially as described previously (26), except that aseptic techniques were used with a laminar flow hood. After collagenase treatment, freshly isolated cells were suspended in culture medium [Joklik minimal essential medium at pH 7.4, supplemented with 1 mM CaCl2, 0.2% (wt/vol) essentially FA-free albumin (30 µM), 1.2 mM MgSO4, 1 mM DL-carnitine, 100 IU/ml penicillin, and 100 µg/ml streptomycin, which had been filtered through a 0.22-µm filter] to a cell density of 150,000 viable cells/ml. Cell number and viability were determined by adding a 30-µl aliquot of resuspended cardiomyocytes to an equal volume of 0.4% (wt/vol) trypsin blue in 0.9% (wt/vol) NaCl. Cell number was determined microscopically in duplicate by counting with a hemacytometer. A myocyte was designated as viable if, on microscopic examination, it was rod-shaped with clear cross striations and excluded trypan blue.

Cardiomyocytes were cultured overnight on laminin-coated plates, using the rapid attachment model of Jacobson and Piper (13). A 2-ml aliquot of culture medium was added to each 35-mm well of laminin-coated six-well tissue culture plates, followed by 1 ml of the freshly isolated myocyte suspension (150,000 viable cells/well). Within 3 h, a large percentage of the myocytes had attached to the laminin-coated wells. At this time, unattached cells and debris were removed from each well by gently aspirating the medium and replacing it with 3 ml of fresh culture medium. The culture plates were incubated at 37°C overnight (18–22 h) in the absence and in the presence of FA under a humidified atmosphere of 95% O2-5% CO2. When FA were added to the
overnight culture medium, appropriate quantities of stock FA solutions (100 mM in hexane) were dried under N₂ gas and resuspended in an equivalent volume of 0.12 M KOH in ethanol. The ethanol was removed by warming under N₂ gas, and the FA (K⁺ salt) were resuspended into the culture medium to give the desired final concentration. The presence of FA in the medium had no effect on the yield or viability (72%) of cardiomyocytes after the overnight (18–22 h) culture. Approximately 50,000 viable cells/well (33% yield) were still attached to the laminin-coated wells after overnight culture. In some experiments, as noted, FA were added with fresh medium after overnight culture in control medium, and the incubation was continued for an additional 1, 3, or 18 h. For measurements of LPL mass, larger numbers of cardiomyocytes (750,000 cells) were cultured overnight, using 100-mm laminin-coated tissue culture dishes.

To determine the fate of oleate added to the overnight culture medium, cardiomyocytes were cultured with 60 µM [¹⁴C]oleate (1 µCi/ml of medium). Triplicate aliquots (10 µl) of FA in the medium had no effect on the yield or viability (72%) of cardiomyocytes after the overnight (18–22 h) culture. Approximately 50,000 viable cells/well (33% yield) were still attached to the laminin-coated wells after overnight culture. In some experiments, as noted, FA were added with fresh medium after overnight culture in control medium, and the incubation was continued for an additional 1, 3, or 18 h. For measurements of LPL mass, larger numbers of cardiomyocytes (750,000 cells) were cultured overnight, using 100-mm laminin-coated tissue culture dishes.

To determine the fate of oleate added to the overnight culture medium, cardiomyocytes were cultured with 60 µM [¹⁴C]oleate (1 µCi/ml of medium). Triplicate aliquots (10 µl) were removed from the culture medium (3-ml initial volume) at various time intervals (0–4 h and 16–18 h), and the radioactivity in these medium samples was measured by liquid scintillation spectrometry. At the end of the overnight culture, the remaining medium was removed and 0.2 ml of 5 N HCl was added to each well. Cells were resuspended by scraping, and the solution was transferred to a test tube. The wells were rinsed with 0.6 ml H₂O, which was added to the scraped cells, and then 4 ml of chloroform-methanol (2:1) were added to the tube. After vortexing and centrifugation, the upper phase was dried under N₂ gas. The extract was dissolved in 50 µl chloroform-methanol (2:1), carrier lipids were added, and lipid classes were separated by thin-layer chromatography (10) with the use of a solvent system consisting of heptane-diethyl ether-glacial acetic acid (25:75:1). Under these conditions, phospholipids remain at the origin; other lipids migrate in the following order from the bottom of the plate: monoacylglycerol, diacylglycerol, FA, and TG (10). Bands corresponding to these lipid classes were identified by I₂ staining, and the content of radioactivity was measured by liquid scintillation spectrometry.

Incubation experiments with heparin were performed with cultured cardiomyocytes to measure LPL activity released into the medium (HR-LPL) and residual cellular LPL (C-LPL) activity. After overnight incubation, the culture medium was removed and replaced with 1 ml of fresh medium containing 5 U/ml heparin (Heparalan) in each well, and the culture plates were returned to the incubator for 40 min. Basal (constitutive) release of LPL into the medium when cardiomyocytes were incubated without heparin was very low. LPL activity in the medium of cultured cardiomyocytes after a 40-min incubation in the absence or in the presence of heparin was (mean ± SE) 18.9 ± 1.6 and 208 ± 18 nmol h⁻¹ mg⁻¹, respectively (n = 60). Thus heparin increased LPL activity in the incubation medium by 11-fold. After incubations with heparin, the medium was removed and centrifuged (15,000 g; Eppendorf microcentrifuge) to collect any dislodged cells. The supernatant (medium) was decanted and frozen for subsequent determination of HR-LPL activity. The cells remaining on the plates were incubated for 20 min at 4°C with 1 ml of a buffer (buffer A) consisting of 0.25 M sucrose, 10 mM N₂-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 1 mM EDTA, and 1 mM dithiothreitol (pH 7.5) and then scraped with a plastic cell scraper. These scraped cells were then added to the cell pellet from the initial medium centrifugation, recentrifuged, as above, and the supernatant was discarded. The cell pellet was then frozen and stored at -80°C. For determinations of C-LPL activity, the frozen cell pellets were resuspended in 0.2 ml 50 mM ammonia buffer (pH 8.0) containing 0.05% Triton X-100 and 0.8 ml of buffer A and then sonicated with a Braun Sonic sonicator at 75 W for two bursts of 30 s at 4°C (9).

LPL assay. LPL activity in the incubation medium (HR-LPL) and in sonicated cell homogenates (C-LPL) from cultured cardiomyocytes was determined by measuring the hydrolysis of a sonicated [³H]triolein substrate emulsion (26), except that the triolein concentration was reduced from 0.6 to 0.1 mM to increase substrate specificity and the sensitivity of the assay. The standard assay contained 0.1 mM glycero-[9,10-³H]triolein (6 mCi/mmol), 25 mM piperaazine-N,N’-bis(2-ethanesulfonic acid) (pH 7.5), 0.05% (wt/vol) essentially FA-free bovine serum albumin (BSA), 50 mM MgCl₂, and 2% (vol/vol) heat-inactivated chicken serum as the LPL activator. A 100-µl aliquot of the incubation medium or 50 µl of the sonicated cell homogenates was then assayed in a final volume of 400 µl. When LPL activity was measured in sonicated cell homogenates, 2 U/ml heparin was added to the assay tubes. The formation of [³H]oleate was measured by liquid-liquid extraction (26), after a 30-min incubation at 37°C. All assays were performed in duplicate, under conditions in which the reaction rate was linear with respect to protein content. LPL activity is expressed routinely as nanomoles of oleate released per hour per milligram protein in the sonicated cell homogenates. Protein concentrations were measured by a Coomassie blue spectrophotometric assay (29), with BSA as the standard. Results are expressed as means ± SE; n refers to the number of wells for which LPL activity was measured. Lipase activity in sonicated cell homogenates was stimulated 4.7-fold by serum (apolipoprotein CII) and was inhibited by >80% by 1 M NaCl, indicating that other TG lipases that may be present in homogenates do not contribute significantly to LPL activity measurements.

Purification of bovine milk LPL and isolation of anti-LPL antibodies. LPL was purified to homogeneity from fresh bovine milk as described by Liu and Severson (18). Purified bovine milk LPL was coupled to Affigel-10 beads (12) for affinity purification of LPL antibodies from chicken eggs. Egg-laying hens were initially injected with 100 µg of bovine milk LPL in complete Freund’s adjuvant in multiple subcutaneous sites on the back (12). Booster injections of 100 µg LPL in incomplete Freund’s adjuvant were given in the thighs and lower neck region at weekly intervals. Five weeks after the initial injection, 15 ml of blood were drawn from the wing veins and eggs were collected.

Immunoglobulin Y (IgY) was isolated, using the water dilution procedure of Akita and Nakai (1). Ten egg yolks from preimmune and immunized hens were diluted sixfold with acidified distilled water (pH 5.2) and then left standing for 5–6 h or overnight. The fluffy solution was then centrifuged for 1 h at 10,000 g at 4°C. The supernatant was collected, and sodium sulfate was added to a final concentration of 19% (wt/vol). After centrifugation at 10,000 g at room temperature, the pellet was resuspended and dialyzed against 10 mM trishydroxymethylaminomethane (Tris) (pH 8) and 0.15 M NaCl (Tris-buffered saline (TBS)); one-third of this water-soluble fraction was applied to a 3-ml LPL-Affigel-10 column. The column was washed with TBS, followed by 10 mM acetate buffer (pH 4.5) and 1 M NaCl. LPL-specific antibody was eluted with 0.2 M glycine-HCl buffer (pH 2.7) and collected in an equal volume of 0.2 M Tris-HCl (pH 8). Total amount of affinity-purified antibody obtained from 10 eggs was ~5 mg; a single band corresponding to chicken IgY was observed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This polyclonal anti-LPL IgY reduces LPL activity (immunoinhibition), and both intact LPL and degra-
lation products are detected by immunoblotting after SDS-PAGE (unpublished results), indicating that the antibody recognizes both active and inactive forms of the enzyme. Control IgY was isolated from preimmune egg yolks.

For biotinylation, the affinity-purified anti-LPL polyclonal antibody (157 µg/ml) in phosphate-buffered saline (PBS) was incubated with 0.1 ml 1 M carbonate buffer (pH 9) and 80 µg N-hydroxysuccinimidyl 6-(biotinamido)hexanoate in dimethylformamide for 90 min at 37°C (12). Excess biotin was removed by dialysis against PBS; biotinylated antibody was stored at –80°C.

Enzyme-linked immunosorbent assay for LPL mass. For measurements of LPL mass, cell pellets were sonicated in 0.2 ml of 25 mM NH₄Cl, 5 mM EDTA, 0.08% (wt/vol) Triton X-100, 0.04% (wt/vol) SDS, 33 µg/ml heparin, and 10 µg/ml leupeptin (pH 8.2). Incubation media were lyophilized and resuspended in 0.2 ml H₂O. A sandwich enzyme-linked immunosorbent assay (ELISA) for LPL mass was developed, using affinity-purified polyclonal antibodies from egg-yelling hens. Polystyrene microtiter plate wells (Immuno 1) were coated with 100 µl of anti-LPL antibody (15 µg/ml in 0.05 M carbonate buffer, pH 9.6) overnight at 4°C. The plates were then washed once with PBS and 0.05% (wt/vol) Tween 20 (buffer B) and blocked for 3 h at 25°C with 3% (wt/vol) BSA containing 3% (vol/vol) FA and 0.1% (wt/vol) sucrose. After four more washes with buffer B, 100-µl aliquots of samples (cell homogenates or incubation media) diluted in PBS containing 0.05% (wt/vol) Tween 20, 1 mg/ml heparin, 0.4% (wt/vol) BSA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 1 µg/ml peptatin A (buffer C) were added to the wells. The plates were then sealed and incubated overnight at 4°C. Purified bovine milk LPL was diazoylated overnight in PBS, diluted in buffer C, and applied to the wells (0.1–1.0 ng) as a standard for the ELISA. After extensive washing, 100 µl of affinity-purified biotin-labeled anti-LPL antibody in PBS containing 1% (wt/vol) BSA were added. After a second overnight incubation at 4°C and washes with buffer B, the wells were incubated with 100 µl of peroxidase-labeled streptavidin in PBS and 1% (wt/vol) FA-free BSA for 2 h at 25°C. The plates were washed four more times, and color reaction was achieved by adding 100 µl of o-phenylenediamine (0.8 mg/ml in 0.15 M citrate buffer, pH 5) and 30 µl of 0.8 M H₂O₂ (buffer D) for 10–15 min. The absorbance at 495 nm was determined with the use of a Bio-Rad microplate reader. LPL mass measurements in cell extracts and in postheparin medium (ng/mg cell protein) were used to calculate LPL specific activity as millimicrons per nanogram LPL protein (9), where 1 mU is defined as the amount of enzyme catalyzing the release of 1 nmol oleate per minute. For LPL specific activity calculations, catalytic activity and mass were measured in the same preparations in which cardiomyocytes cultured overnight in the presence of FA were paired with control cultured cells.

Immunohistochemistry. Cultured cardiomyocytes were resuspended into PBS (pH 7.4) containing 1% (wt/vol) FA-free albumin at 37°C. After centrifugation, the cell pellet was resuspended into cold PBS containing 0.625 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid and 2.5 mM NaOH for 5 min. Cells were then fixed with 2.5% Formalin in PBS and finally stored in PBS at a concentration of 10⁶ cells/ml. Aliquots (25 µl) of this fixed but nonpermeabized cell suspension were then incubated with 2 µg/ml preimmune IgY or affinity-purified anti-LPL IgY in PBS containing 3% albumin and 0.05% Tween (dilution buffer) overnight at 4°C. After washing, rhodamine [tetramethylrhodamine isothiocyanate (TRITC)]-conjugated rabbit anti-chicken IgY was added to the cells at 1:50 dilution in dilution buffer and incubated for 1 h at 4°C in the dark. The cells were then washed extensively and resuspended in 10–20 µl PBS. A 6-µl aliquot was mounted on a slide in 90% glycerol in PBS. The slides were viewed on an Olympus BH2-RFCA fluorescence microscope, and black and white pictures were recorded on Kodak T-max 400 film.

Enzyme-linked immunosorbent assay. An aliquot (4.5 ml) of the postheparin medium from freshly isolated cardiomyocytes (2 x 10⁶ cells/ml incubated with 5 U/ml heparin for 1 h) was added to 1 ml heparin-Sepharose (7). After a 30-min incubation at 4°C, the mixture was poured into a column, washed with 20 ml of 20 mM HEPES (pH 7.4), 20% (vol/vol) glycerol, and 0.02% Triton X-100 (buffer D), and the flow-through fractions (2 ml each) were collected. The column was then washed with successive additions of buffer D containing increasing concentrations of NaCl (0.05, 0.6, and 1.5 M NaCl). Fractions were assayed immediately for LPL activity. Because of the presence of variable amounts of NaCl in the column fractions, 50 mM MgCl₂ was omitted from the assay, and the final NaCl concentration in the assay was kept constant at 0.28 M.

Materials. Collagenase ( Worthington type II) was obtained from Technicon Canada (Richmond, BC, Canada), and heparin (Heparalean; 100 U/ml) was purchased from Organon Teknika (Toronto, ON, Canada). J ořík minimal essential medium, Dulbecco’s modified Eagle’s medium (DMEM) (high and low glucose), and penicillin/streptomycin were purchased from Gibco Canada (Burlington, ON, Canada). [³H]triolein (glycerol [9,10-³H]trioleate) and [1-¹⁴C]oleic acid were purchased from Amersham (Oakville, ON, Canada). Tissue culture plates (6 well; Falcon) were coated with 15 µg/ml laminin in Hank’s balanced salt solution for 3 h at 37°C; the plates were then air dried and stored at 4°C until use. Freund’s adjuvants, 4% Formalin, oxefunic, and ultrapure mouse laminin were purchased from Sigma Chemical (St. Louis, MO). Rhodamine (TRITC)-conjugated rabbit anti-chicken IgY was obtained from BioCan Scientific (Mississauga, ON, Canada), and peroxidase-labeled streptavidin was from Boehringer Mannheim (Laval, PQ, Canada). FA were purchased from either Sigma or Serday Research Laboratories (London, ON, Canada). All other chemicals were from either Sigma or VWR Scientific of Canada (Edmonton, AB, Canada).

RESULTS

LPL activity in cultured myocytes. Total C-LPL activity was unchanged after overnight culture, but HR-LPL activity was increased by 3.4-fold in cultured cardiomyocytes compared with freshly isolated cells. Presumably, this selective increase in HR-LPL activity reflects the replenishment of LPL and/or LPL binding sites on the cell surface after collagenase treatment. Effect of glucose concentration on LPL activity in cultured cardiomyocytes. Overnight culture of cardiomyocytes with varying glucose concentrations (5, 6, 11, and 25 mM) resulted in no significant differences in either HR-LPL or C-LPL activities (Fig. 1).

Effect of FA on LPL activity in cultured cardiomyocytes. In contrast to the preceding results with glucose, the addition of oleate to the overnight culture medium produced a concentration-dependent inhibition of HR-LPL activity (Fig. 2), with no significant change in C-LPL activity. The heparin-containing medium from oleate-treated cells did not reduce control HR-LPL activity in mixing experiments, indicating that an inhibitor was not released into the medium when cardiomyocytes cultured in the presence of oleate were
incubated with heparin. Because considerable inhibition of HR-LPL activity was observed with 60 µM oleate (2:1 molar ratio to the albumin concentration in the culture medium), this concentration was used in subsequent experiments.

Total LPL mass in control cells was 38.4 ± 10.7 ng/mg protein (n = 4 preparations of cultured cardiomyocytes). LPL mass in the medium of undetected cells incubated in the absence of heparin was undetectable, consistent with the low level of LPL activity measured as an index of constitutive LPL release. In the presence of heparin, LPL mass in the medium of cultured cardiomyocytes was 8.4 ± 1.6 ng/mg cell protein (n = 6). Thus heparin released ~20% of total cellular mass into the medium, but C-LPL and HR-LPL activities were very similar (Figs. 1 and 2), indicating that heparin released ~50% of total cellular enzyme activity. This apparent discrepancy is due to the presence of a substantial quantity of inactive enzyme mass in cultured cardiomyocytes; LPL specific activities in cell extracts and heparin-treated medium were 0.075 ± 0.008 and 0.72 ± 0.15 mU/ng LPL protein, respectively. Thus heparin must preferentially release active dimeric LPL into the medium of control cells.

Overnight culture with 60 µM oleate did not significantly alter LPL mass in cells (42.1 ± 7.7 ng/mg cell protein; n = 4) or in the medium after incubation with heparin (5.6 ± 0.5 ng/mg protein; n = 6) compared with mass determinations in control cultured cardiomyocytes. As a consequence, the specific activity of HR-LPL (mU/ng LPL protein) was reduced from 0.72 ± 0.15 to 0.44 ± 0.06 (P < 0.05) by the overnight incubation with oleate.

The effect of 60 µM concentrations of different FA in the overnight culture medium on LPL activity is shown in Fig. 3. Palmitate (16:0) and myristate (14:0), like oleate (18:1), produced a marked reduction in HR-LPL activity without any significant change in C-LPL activity. Although linoleate (18:2) and eicosapentaenoate (20:5) did significantly reduce C-LPL activity to 57 and 52% of control, respectively (Fig. 3), HR-LPL activity was inhibited to a much greater extent (to 15 and 9% of control, respectively). Thus all FA resulted in a selective inhibition of HR-LPL activity.

The fate of 60 µM oleate during the overnight culture was examined next. When [14C]oleate was added to the culture medium, there was a time-dependent decrease in medium radioactivity to ~40–50% of control (zero time) after 18 h (Fig. 4). When cardiomyocytes were extracted after overnight culture with [14C]oleate, most of the radioactivity incorporated into cellular lipids was recovered in TG (50 ± 1% of total radioactivity in the lipid extract; n = 3) and phospholipid (36 ± 4%). Oxfenicine (100 µM), an FA oxidation inhibitor (30), did not alter the inhibition of HR-LPL activity in cultured cardiomyocytes by 60 µM oleate (Fig. 5).

Even though LPL mass displaced into the medium by heparin was not reduced by prior incubation with oleate, additional experiments were conducted to determine whether FA (oleate) displaced LPL, bound to HSPG on the cell surface of cardiomyocytes (6, 7, 21), into the medium during the overnight culture, thus reducing HR-LPL activity measured in the subsequent
40-min incubation with heparin. LPL activity in the medium after overnight culture in the absence and in the presence of 60 µM oleate was 15 ± 3 (n = 12) and 3 ± 1 (n = 12) nmol·h⁻¹·mg⁻¹, respectively. However, any LPL displaced by oleate likely would have been inactivated during the long (18–22 h) incubation time at 37°C. Therefore, the effect of oleate added to cardiomyocytes after overnight culture on LPL activity in the medium and on HR-LPL activity was determined (Table 1). Incubation of cultured cardiomyocytes with 60 µM oleate for 1 h did not alter either medium LPL activity or HR-LPL activity. In contrast, the addition of heparin to the 1-h incubation displaced LPL into the medium and consequently reduced HR-LPL activity in the subsequent incubation with heparin (Table 1). Increasing the incubation time with 60 µM oleate to 3 h still produced no increase in medium LPL activity, even though HR-LPL activity was reduced significantly. Incubation of control overnight-cultured cardiomyocytes with 60 µM oleate for an additional 18 h reduced HR-LPL activity to 8 nmol·h⁻¹·mg⁻¹ (Table 1), compared with control activity of 120 ± 19 nmol·h⁻¹·mg⁻¹.

Fig. 3. Effect of fatty acids (FA) on LPL activity in cultured cardiomyocytes. Cardiomyocytes were cultured overnight in absence (open bars) and in presence (solid bars) of the following FA (all at 60 µM): palmitate (16:0), myristate (14:0), oleate (18:1), linoleate (18:2), and eicosapentaenoate (20:5). Fresh culture medium containing 5 U/ml heparin was then added to each well, and HR-LPL (A) and C-LPL (B) activities were measured. Results are means ± SE for number of wells indicated in parentheses. *P < 0.05; **P < 0.01; ***P < 0.001 (Student’s t-test).

Fig. 4. Utilization of [¹⁴C]oleate by cultured cardiomyocytes. Cardiomyocytes were cultured overnight with 60 µM [¹⁴C]oleate (1 µCi/ml culture medium). At indicated times, triplicate 10-µl aliquots of medium were removed, and radioactivity was measured. Results are from 2 separate preparations (○ and △) of cultured cardiomyocytes.

Fig. 5. Effect of oxfenicine on inhibition of HR-LPL activity in cultured cardiomyocytes by oleate. Cardiomyocytes were cultured overnight in presence of no additions, 100 µM oxfenicine, 60 µM oleate (18:1), and oxfenicine plus oleate. Results are means ± SE for number of wells indicated in parentheses.
Table 1. Effect of oleate on medium LPL and HR-LPL activities in cultured cardiomyocytes

<table>
<thead>
<tr>
<th>Additions After</th>
<th>Incubation Time, h</th>
<th>Medium LPL, nmol·h⁻¹·mg⁻¹</th>
<th>HR-LPL, nmol·h⁻¹·mg⁻¹</th>
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<tr>
<td>Overnight Culture</td>
<td></td>
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<tr>
<td>None</td>
<td>0</td>
<td>201 ± 13 (61)</td>
<td></td>
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<tr>
<td>None</td>
<td>1</td>
<td>8 ± 2 (6)</td>
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</tr>
<tr>
<td>Oleate (60 µM)</td>
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<td>3 ± 1 (12)</td>
<td>167 ± 14 (15)</td>
</tr>
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<td>Heparin (5 U/ml)</td>
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<td>91 ± 5 (6)</td>
<td>43 ± 7* (3)</td>
</tr>
<tr>
<td>Oleate (60 µM)</td>
<td>3</td>
<td>8 ± 2 (12)</td>
<td>109 ± 7† (14)</td>
</tr>
<tr>
<td>Oleate (60 µM)</td>
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<td>24 ± 10 (6)</td>
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</tr>
<tr>
<td>None</td>
<td>18</td>
<td>120 ± 19 (12)</td>
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</table>

Results are means ± SE for number of culture wells in parentheses. Cardiomyocytes were cultured overnight under control conditions (no additions), and heparin-releasable lipoprotein lipase (HR-LPL) activity was measured at zero time. Cultured cardiomyocytes were then incubated in fresh medium with indicated additions for 1, 3, or 18 h; medium was removed and LPL activity was measured. Then, fresh culture medium containing 5 U/ml heparin was added, and HR-LPL activity was measured after a 40-min incubation. *P < 0.05; †P < 0.01; and ‡P < 0.001 (Student’s t-test) relative to control (no additions) HR-LPL activity.

when cells were cultured for another 18 h without oleate. This inhibitory effect was similar to the reduction in HR-LPL activity observed when oleate was present in the initial overnight culture medium (Figs. 2 and 3), but LPL activity in the medium was still not increased (Table 1).

When LPL activity was measured in the medium of cultured cardiomyocytes after incubation with 60 µM oleate for 1 or 3 h (Table 1), the final assay incubation contained 15 µM oleate (100 µl of medium assayed in a total volume of 400 µl; see MATERIALS AND METHODS). The direct addition of 15 µM oleate to assays with control medium from heparin-treated cardiomyocytes did not reduce HR-LPL activity (119 and 125 nmol·h⁻¹·mg⁻¹ in the absence and presence of 15 µM oleate, respectively). Therefore, LPL activity in the medium of oleate-treated cells is not masked by interference of FA introduced into the assay.

LPL activity released into the medium of cultured cells by heparin is somewhat unstable at 37°C; therefore, the possibility that oleate increases the rate of LPL inactivation in the medium was examined. As shown in Fig. 6A, incubation of the medium of heparin-treated cultured cardiomyocytes for up to 60 min at 37°C resulted in a time-dependent decrease in HR-LPL activity. However, this inactivation was reduced, not enhanced, when 60 µM oleate was present (Fig. 6B).

The reversibility of the inhibitory effect of oleate on HR-LPL activity was also examined. Cardiomyocytes were first cultured overnight with oleate to reduce HR-LPL activity (to 31 ± 5 nmol·h⁻¹·mg⁻¹; n = 9). The cells were then cultured for an additional 18 h in fresh medium containing no oleate; HR-LPL activity increased to 60 ± 6 nmol·h⁻¹·mg⁻¹ (n = 15), relative to 120 ± 19 nmol·h⁻¹·mg⁻¹ in control cardiomyocytes cultured continuously in the absence of oleate (Table 1). Thus the inhibitory effect of oleate on HR-LPL is at least partially reversible in this time frame.

The possibility that the binding of FA to LPL (11) might alter the ability of the enzyme to bind to heparin was tested by subjecting postheparin medium (HR-LPL) to heparin-Sepharose chromatography (Fig. 7). LPL binds tightly to heparin-Sepharose so that high ionic strength is required to displace the enzyme (7). The presence of 60 µM oleate in the postheparin medium had no effect on the binding of LPL to the column or on its elution with 1.5 M NaCl (Fig. 7).

Histochemical detection of LPL on the cell surface of cultured cardiomyocytes. The detection of LPL on the surface of nonpermeabilized cultured cardiomyocytes by immunofluorescence, using an affinity-purified chicken polyclonal antibody to LPL, was examined next. A typical pattern of immunostaining with control cultured cardiomyocytes is shown in Fig. 8A; the most intense fluorescent labeling was observed at the edges of cells. Surprisingly, cardiomyocytes after overnight culture with 60 µM oleate consistently exhibited more immunodetectable LPL on the cell surface (Fig. 8B) compared with control cultured cardiomyocytes.

![Fig. 6. Stability of HR-LPL activity. Cardiomyocytes were cultured overnight under control conditions. Fresh medium containing 5 U/ml heparin was added, and after a 40-min incubation, medium was collected for determination of HR-LPL activity. A: heparin-treated medium was incubated at 37°C for indicated times, and HR-LPL activity was measured. Results are means of 2 experiments with different cultured cardiomyocyte preparations. B: HR-LPL activity was measured in medium at zero time and after 40-min incubation at 37°C with either no additions (open bars) or with 60 µM oleate added to medium (solid bars). Results are means ± SE (n = 7 incubations).](http://altpendoproxy.org/...)
DISCUSSION

Kern et al. (15) reported that increasing the glucose concentration in the culture medium of isolated human adipocytes from 5.5 to 25 mM reduced the basal constitutive release of LPL activity by 50%. The basal or constitutive release of LPL into the medium of cultured cardiomyocytes is very low and was unaffected at different glucose concentrations (results not shown). Variation of the glucose concentration in the culture medium (5.5, 11, and 25 mM) also had no significant effect on either HR-LPL or C-LPL activities in cultured cardiomyocytes, suggesting that the hyperglycemia that accompanies diabetes does not regulate myocardial LPL activity.

A number of investigations have shown that functional endothelium-bound LPL activity is regulated acutely by FA to prevent the excessive delivery of lipolytic products to tissue cells. First, FA inhibit LPL activity directly by product inhibition and by reducing the activation by apolipoprotein CII (3); LPL has four to six FA binding sites (11). Second, FA can displace LPL from binding sites on the surface of cultured endothelial cells (27). Thus this latter mechanism can account for the observation that infusion of a TG emulsion to humans (24) or administration of an oral fat load (14) increased plasma LPL activity. These acute mechanisms for regulating endothelium-bound LPL may be inadequate under certain circumstances, such as the chronic increases in plasma FA that occur in diabetes, so it is reasonable to anticipate that FA may also regulate the activity of LPL in parenchymal cells (e.g., cardiomyocytes and adipocytes).

Addition of a low concentration of oleate (60 µM) at a physiological FA-to-albumin ratio (2:1) to the overnight culture medium produced a profound decrease in HR-LPL activity to 20% of control but no significant change in residual C-LPL activity (Fig. 2) or in C-LPL mass. This selective inhibitory effect on HR-LPL activity was also seen with saturated FA, palmitate, and myristate. Although linoleate and eicosapentaenoate did result in a significant decrease in C-LPL activity, the reduction in HR-LPL activity was much greater (Fig. 3).

The selective inhibition of HR-LPL activity in cultured cardiomyocytes by FA is similar to results obtained by Amri et al. (2) with cultured Ob 1771 adipocytes, in which FA produced a greater decrease in HR-LPL activity compared with C-LPL activity, with no change in LPL mass measured by immunoblotting. On the other hand, different results were obtained with cultured rat adipocyte precursors (16) and cultured chicken adipocytes (19), in which FA in the culture medium decreased LPL activity in cell extracts by a transcriptional mechanism, with reductions in LPL mRNA and LPL synthesis/mass (16, 19). Therefore, the mechanism of FA inhibitory effects on LPL activity can vary markedly, depending on the cell system.

The inhibitory effect of oleate on HR-LPL activity in cultured cardiomyocytes was not altered by oxfenicine (Fig. 5), an inhibitor of FA oxidation (30). This is perhaps not surprising, since cultured cardiomyocytes are quiescent and have low rates of FA oxidation (25);
esterification to form endogenous TG is the principal metabolic fate for FA added to the medium of cultured cardiomyocytes. Amri et al. (2) observed that nonmetabolized FA analogs were capable of reducing LPL activity in cultured Ob 1771 adipocytes.

LPL bound to HSPG on the cell surface of cultured cardiomyocytes could have been displaced into the culture medium by FA, as shown with cultured endothelial cells (27). This FA-induced displacement could then produce the observed selective reduction in HR-LPL activity. This potential mechanism seems unlikely, however, for the following reasons. First, displacement of cell surface LPL by FA should be rapid; the addition of oleate to cultured endothelial cells released 80% of bound LPL into the medium during a 1-h incubation at 37°C (27). By comparison, a 1-h incubation of cultured cardiomyocytes with oleate resulted in no increase in medium LPL activity (Table 1). LPL activity potentially present in the medium was not masked by the transfer of inhibitory amounts of oleate into the LPL assay, and oleate did not accelerate the inactivation of LPL in the culture medium. In addition, any displacement of LPL into the culture medium during the 1-h incubation, even if not detected by direct assay of enzyme activity, should also reduce HR-LPL activity as shown with heparin (Table 1), but the incubation with oleate for 1 h did not reduce HR-LPL activity. A small reduction in HR-LPL activity was seen after the 3-h incubation with oleate, but no increase in medium LPL could be detected. Therefore, the time course for the inhibitory effect of oleate on HR-LPL (3–18 h) is inconsistent with a displacement mechanism. Furthermore, the mass of LPL displaced into the medium by heparin was not reduced after overnight culture with oleate, and more immunodetectable LPL (rather than less) was evident on the cell surface of oleate-treated cardiomyocytes compared with control cultured cells (Fig. 8). These results with cultured cardiomyocytes are consistent with a previous investigation from our laboratory that reported that FA did not release LPL from binding sites on the coronary vasculature of perfused hearts or from the surface of freshly isolated cardiomyocytes (26).

Other mechanisms therefore must account for the selective reduction in HR-LPL activity when FA are added to the medium of cultured cardiomyocytes. For example, the processing of LPL (7) could be affected by FA so that more inactive monomeric but immunodetectable LPL is present on the cell surface. Because the overnight culture with FA did not change total C-LPL mass measured by ELISA, FA must stimulate the transport of LPL from intracellular compartments to the cell membrane of cultured cardiomyocytes. As a result, the relative proportion of inactive to active forms of LPL on the cell surface may be increased by FA treatment to account for the observation that heparin released the same total mass of LPL (active and inactive enzyme) into the medium of oleate-treated cells, even though HR-LPL activity was reduced substantially. The acute presence of FA in postheparin medium did not alter binding of LPL to heparin-Sepharose (Fig. 7). Nevertheless, cell surface binding sites (HSPG) for LPL may be altered by chronic exposure of cardiomyocytes to FA to impair LPL displacement by heparin, since oleate treatment did not change LPL mass displaced into the medium by heparin, even though the total immunodetectable LPL mass on the cell surface was increased considerably (Fig. 8). Diabetes alters sulfation of HSPG in hepatocytes (31); FA may produce similar changes in HSPG composition in cultured cardiomyocytes and thus influence LPL binding. The constitutive (basal) release of LPL activity is extremely low, so FA are unlikely to produce the increase in cell surface immunodetectable LPL by somehow stabilizing binding of LPL to reduce constitutive secretion into the medium.

In summary, FA selectively reduced HR-LPL activity in cultured cardiomyocytes, despite an increase in immunodetectable LPL on the cell surface. The FA-induced impairment of the heparin-induced release of LPL into the medium may be due to alterations in the intracellular processing of LPL and the subsequent movement of the enzyme to the cell membrane, together with changes in cell surface binding sites. More investigations are required to determine if this inhibitory effect of FA on HR-LPL activity could contribute, at least partially, to the diabetes-induced reduction in LPL activity in cardiomyocytes.

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Address for reprint requests: D. L. Severson, The University of Calgary, Faculty of Medicine, Department of Pharmacology and Therapeutics, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1.

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