Lipoprotein lipase activity in rat cardiomyocytes is stimulated by insulin and dexamethasone

H. Stephen EWART, Rogayah CARROLL and David L. SEVERSON¹

The University of Calgary, Department of Pharmacology and Therapeutics, 3330 Hospital Drive N. W., Calgary, AB, T2N 4N1, Canada

Lipoprotein lipase (LPL) activity was studied in rat cardiomyocytes after overnight culture (16 h) in the presence of insulin (100 nM) and/or dexamethasone (100 nM). Insulin in combination with dexamethasone (INS/DEX) increased heparin-releasable LPL activity by 71 % over the control level (566 ± 85 versus 331 ± 48 nmol/h · mg cell protein). This was accompanied by a 61 % increase in total cellular LPL activity (914 ± 89 versus 567 ± 64 nmol/h · mg cell protein). The increase in LPL activity

occurred at sub-nanomolar concentrations of the hormones, but neither hormone was effective alone. LPL protein mass, quantified by ELISA, was the same in both control and INS/DEX-treated cells (27.7 versus 28.6 ng/mg cell protein, respectively), thus LPL specific activity in cardiomyocytes was increased by INS/DEX treatment (0.113 versus 0.069 mU/ng LPL protein). These findings emphasize the importance of hormonal interactions in the regulation of LPL in heart tissue.

INTRODUCTION

Lipoprotein lipase (LPL) is a glycoprotein (EC 3.1.1.34) that catalyses the hydrolysis of triacylglycerol to free fatty acids [1]. In heart, the enzyme is synthesized in cardiomyocytes and then secreted and translocated to the coronary vasculature where it functions in the clearance of lipoprotein triacylglycerol from the circulation [1–3]. The fatty acids released by the catalytic activity of LPL are used by cardiomyocytes as an oxidative fuel.

The hormonal regulation of LPL in heart tissue has not been well characterized. During fasting, LPL activity in heart is increased [1], whereas in the case of diabetes, myocardial LPL activity is decreased [4–6]. Insulin has been shown to increase LPL activity in isolated cardiomyocytes after injection into control and diabetic rats [4,5]. However, attempts to demonstrate a stimulatory response *in vitro* by incubating freshly isolated cardiomyocytes with this hormone have been unsuccessful [5]. This may not be attributable to damage to insulin receptors caused during the collagenase treatment to isolate cardiomyocytes, since other parameters, including glucose oxidation and protein synthesis, are still stimulated by insulin in these cells [5]. It is possible, therefore, that an additional *in vivo* factor, such as another hormone, may have to act with insulin to stimulate myocardial LPL.

The present study was designed to determine whether LPL activity was increased in cultured cardiomyocytes after an overnight incubation with insulin. The effect of chronic insulin exposure *in vitro* on heart LPL activity has not been previously investigated. Since glucocorticoids augment the stimulation of LPL by insulin in adipose tissue [7–9], we also examined whether insulin in combination with a glucocorticoid (dexamethasone) would have a stimulatory effect on LPL activity in cultured cardiomyocytes.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats (180–200 g) were obtained from local breeding sources at the University of Calgary. Joklik minimal

essential medium and penicillin/streptomycin were purchased from Gibco Canada (Burlington, ON). Collagenase was purchased from Technicon Canada Ltd. (Richmond, BC), heparin (1000 units/ml; Hepalean) was obtained from Organon Teknika (Toronto, ON) and laminin was from Becton Dickinson Labware (Bedford, MA). Insulin, dexamethasone and essentially fatty-acid-free BSA were products of Sigma Chemical Co. (St. Louis, MO). Glycerol-[9,10-³H]trioleate was from Amersham Canada (Oakville, ON).

Preparation and incubation of cardiomyocytes

Ventricular cardiomyocytes were isolated essentially as described previously [10], but under aseptic conditions. Following collagenase treatment, the cells were resuspended in culture medium (Joklik minimal essential medium supplemented with 25 mM NaHCO₃, 1 mM CaCl₂, 1.2 mM MgSO₄, 1 mM D,L-carnitine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.2 % (w/v) BSA, pH 7.4) which had been filtered through a 0.22 µm filter. Cell viability was determined by Trypan Blue (0.4 % in 0.9 % NaCl) exclusion. Viable cells had a rod-shaped morphology with clear cross-striations. Cell numbers were determined in duplicate using a haemocytometer. A preparation from a single adult rat heart yielded routinely (7–9) × 10⁶ viable cells, which were diluted to a density of 150 000 cells/ml.

Cardiomyocytes were cultured using the rapid-attachment model of Jacobson and Piper [11]. For measurements of LPL activity, cells were cultured in laminin-coated 6-well (35-mm) plates; 2 ml of culture medium was added to each well, followed by 1 ml of the myocyte suspension. For measurements of LPL mass, 5 ml of myocyte suspension was added to 10 ml of culture medium in laminin-coated 10-cm culture dishes. Fresh medium was provided after 3 h which contained various additions: insulin (100 nM), dexamethasone (100 nM), or insulin in combination with dexamethasone (INS/DEX). Control incubations received an equal volume of vehicle (1 µl/ml 10 mM HCl). Cells were incubated overnight (16 h) at 37 °C under a humidified atmosphere of 95 % O₂/5 % CO₂. Following this treatment, the cells were incubated in fresh culture medium (1 ml/well or

Abbreviations used: LPL, lipoprotein lipase; INS/DEX, insulin in combination with dexamethasone; HR-LPL, heparin-releasable lipoprotein lipase; C-LPL, cellular lipoprotein lipase; TBS, 10 mM Tris/0.15 M NaCl (pH 8.0).

¹ To whom correspondence should be addressed.

2 ml/10-cm dish) with or without 5 U/ml heparin for 30 min at 37 °C. The medium from heparin-treated cells was removed and frozen at -80 °C for subsequent determinations of heparin-releasable LPL (HR-LPL) activity. HR-LPL activity represents the fraction of total cellular LPL that is bound to the cell-surface of cardiomyocytes [5]. Total cellular LPL activity (C-LPL) was determined in extracts from cardiomyocytes incubated in the absence of heparin. Cells were scraped in lysis buffer (50 mM ammonia buffer, pH 8.0, containing 0.05 % Triton X-100), sonicated and stored at -80 °C [12].

Assay of LPL activity

LPL activity in the incubation medium (HR-LPL) and in sonicated cell lysates (C-LPL) was determined by measuring the hydrolysis of a sonicated [³H]triolein substrate emulsion [10]. The assay contained 0.1 mM glycerol-[9,10-³H]trioleate (6 mCi/mmol), 25 mM Pipes (pH 7.5), 0.05 % essentially fatty-acid-free BSA, 50 mM MgCl₂ and 2 % heat-inactivated chicken serum as the LPL activator. For cell lysates, 2 U/ml heparin was also present. A 100 µl aliquot of incubation medium or 50 µl of cell lysate was assayed in a final volume of 400 µl. The formation of [³H]oleate was measured by liquid-liquid extraction [10] following a 30 min incubation at 30 °C. All assays were performed in duplicate; activity is expressed routinely as nmol of oleate released/h·mg protein in the sonicated cell extracts. Protein concentration was measured using a Coomassie Blue spectrophotometric assay [13] with BSA as the standard.

Preparation of anti-LPL antibodies

LPL was purified to homogeneity from fresh bovine milk as described by Liu and Severson [14]. Egg-laying hens were initially injected with 100 µg of purified LPL in complete Freund's adjuvant in multiple subcutaneous spots along the back [15]. Booster injections of 100 µg of purified 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 was drawn from the wing veins and eggs were collected.

Purified bovine milk LPL was coupled to Affigel-10 beads [15] for affinity purification of LPL antibodies. IgY was isolated using the water-dilution procedure of Akita and Nakai [16]. Ten yolks from preimmune and immunized hens were diluted 6-fold with acidified distilled water (pH 5.2) and left standing either 5–6 h or overnight. The fluffy solution was centrifuged for 1 h at 10000 g, 4 °C. The supernatant was collected and sodium sulphate was added to a final concentration of 19 % (w/v). After centrifugation at 10000 g at room temperature, the pellet was resuspended and dialysed against 10 mM Tris/0.15 M NaCl, pH 8.0 (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), 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 8.0). Total affinity-purified antibody obtained from 10 eggs was about 5 mg; a single band corresponding to chicken IgY was observed after SDS/PAGE. Control IgY was isolated from preimmune egg yolks.

For biotinylation, the affinity-purified anti-LPL antibody (157 µg) in 1 ml of PBS was incubated with 0.1 M NaHCO₃ buffer (pH 9.0) and 80 µg *N*-hydroxysuccinimidyl(6-biotin-amido)hexanoate in dimethyl formamide for 90 min at 37 °C [15]. Excess biotin was removed by dialysis against PBS. Biotinylated antibody was stored at -80 °C.

ELISA for LPL

Following 30 min incubations with and without heparin, media from 10-cm dishes were removed, freeze-dried and resuspended in 0.2 ml H₂O. Cells were scraped in 1 ml of buffer (250 mM sucrose/20 mM Hepes/1 mM EDTA/1 mM dithiothreitol/pH 7.4) and pelleted. The pellet was sonicated in 0.2 ml of 25 mM NH₄Cl, 5 mM EDTA, 0.8 % (w/v) Triton X-100, 0.04 % (w/v) SDS, 33 µg/ml heparin and 10 µg/ml leupeptin (pH 8.2). Samples were diluted for subsequent assay in PBS containing 0.05 % Tween 20, 1 mg/ml heparin, 0.4 % BSA, 1 mM PMSF, 10 µg/ml leupeptin and 1 µg/ml pepstatin A (pH 7.5).

An ELISA for LPL was developed using affinity-purified polyclonal antibodies from egg-laying hens. Polystyrene microtitre plate wells (Immulon 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 (0.05 % Tween 20) and blocked for 3 h at 25 °C with 300 µl of PBS containing 3 % BSA (w/v) and 0.1 % (w/v) sucrose. After four washes with PBS (0.05 % Tween 20), aliquots of samples (1, 2 and 3 µl of cell lysates or 2, 4 and 8 µl of incubation media in a total volume of 100 µl) were added to the wells. Purified bovine milk LPL [14] that had been dialysed overnight in PBS was diluted in the same buffer as the samples were and added to wells (0.1–1.0 ng) to serve as standards for ELISA. The plates were sealed and incubated overnight at 4 °C. After several washes, 100 µl of affinity-purified anti-LPL antibody (in PBS containing 1 % BSA) which was biotin-labelled was added. This was incubated overnight at 4 °C, washed several times and incubated with 100 µl of peroxidase-labelled streptavidin in PBS (1 % BSA) for 2 h at 25 °C. Following four washes, a colour reaction was initiated by adding 100 µl of *o*-phenylenediamine (0.8 mg/ml in 0.15 M citrate buffer, pH 5) and 0.003 % (v/v) H₂O₂. After development for 10–15 min, the absorbance at 495 nm was determined using a Biorad microplate reader. LPL mass is expressed as ng/mg cell protein; LPL specific activity is expressed as mU/ng LPL protein, where 1 mU is defined as the amount of enzyme catalysing the release of 1 nmol oleate/min [12]. This ELISA method appears to detect both monomeric and dimeric forms of the enzyme, as determined by incubating the medium from heparin-treated cells at 45 °C for 1 h to inactivate the enzyme through dissociation of enzyme subunits [17]. The heat treatment did not significantly affect LPL mass detected by the above method (15.8 versus 12.1 ng/ml for control and heat-treated medium, respectively), but markedly reduced LPL activity (731 versus 10 nmol/h·ml medium for control and heat-treated medium, respectively).

Statistics

Results are expressed as means ± S.E.M., where *n* is the number of individually cultured cardiomyocyte preparations. The effect of the various hormone treatments on HR-LPL and C-LPL activities was analysed using one-way analysis of variance (ANOVA), with comparisons made by the Student–Newman–Keuls multiple-comparisons test (*P* < 0.05 indicating statistical significance). Data shown in Table 2 were compared using Student's paired *t* test (*P* < 0.05 indicating statistical significance).

RESULTS AND DISCUSSION

Preliminary experiments with freshly isolated cardiomyocytes showed that short-term incubations (2 h) with insulin or INS/DEX did not increase either HR-LPL or C-LPL activities (results not shown). The effect of these hormones on LPL activity after

Table 1 The effect of insulin and dexamethasone on total cellular LPL and heparin-releasable LPL activities in cultured cardiomyocytes

Cardiomyocytes were cultured overnight in either the absence or in the presence of 100 nM insulin (INS), 100 nM dexamethasone (DEX) or insulin plus dexamethasone (INS/DEX). Total LPL activity was measured in lysates of cells not treated with heparin (C-LPL) and in incubation medium from heparin-treated cells (HR-LPL). The results are means $\pm$ S.E.M. of 12 separate experiments. Comparisons were made using Student–Newman–Keuls multiple comparisons test ($P < 0.05$).

Additions	LPL activity (nmol/h $\cdot$ mg cell protein)	
	C-LPL	HR-LPL
None	567 $\pm$ 64	331 $\pm$ 48
INS	646 $\pm$ 73†	373 $\pm$ 47†
DEX	688 $\pm$ 68†	364 $\pm$ 65†
INS/DEX	914 $\pm$ 89*	566 $\pm$ 85*

†Significantly different from incubation with INS/DEX.

* Significantly different from incubation with no additions.

overnight (16 h) culture was therefore investigated. Neither insulin nor dexamethasone alone had a significant effect on either C-LPL or HR-LPL activities (Table 1). However, when the hormones were added together, C-LPL activity was increased by 61 % (914 $\pm$ 89 versus 567 $\pm$ 64 nmol/h $\cdot$ mg cell protein) and HR-LPL activity was increased by 71 % (566 $\pm$ 85 versus 331 $\pm$ 48 nmol/h $\cdot$ mg cell protein) (Table 1).

A stimulatory effect of INS/DEX was also observed on residual cellular LPL activity following heparin treatment (415 $\pm$ 49 versus 307 $\pm$ 40 nmol/h/mg cell protein; $p < 0.05$, paired t test). Thus the increase in total C-LPL activity elicited by INS/DEX involves an increase in a component of total cellular LPL activity that is not released by heparin, in addition to increased activity in the heparin-releasable fraction. It should also be noted that the sum of HR-LPL activity and the residual cellular LPL activity (following heparin treatment) consistently exceeded total C-LPL by 30–40 %, and this was not affected by any of the hormonal treatments. The mechanism for this discrepancy is unknown, but ongoing LPL synthesis and release during the heparin treatment is a contributing factor.

Figure 1 shows the sensitivity of the stimulation of HR-LPL activity by insulin and dexamethasone. Both hormones were effective in the sub-nanomolar range. In the presence of 100 nM insulin, HR-LPL activity was increased by 45 % at 10^{-9} M DEX (Figure 1a), with a maximal stimulation of 64–66 % at 10^{-5} – 10^{-4} M. Conversely, 10^{-9} M insulin produced a maximal stimulatory effect on HR-LPL activity in the presence of 100 nM dexamethasone (Figure 1b). These observations suggest that both insulin and glucocorticoids are relevant physiologically in the regulation of LPL activity in heart tissue.

To investigate the molecular basis of the stimulatory effect of INS/DEX on LPL activity in cultured cardiomyocytes, LPL mass was measured using the ELISA technique. Overnight treatment with INS/DEX did not alter LPL mass measured in cellular lysates (Table 2). Consequently, enzyme specific activity, calculated by dividing LPL activity by the corresponding mass value, was increased ($P < 0.01$) from 0.069 to 0.113 mU/ng LPL protein by INS/DEX treatment. This suggests that a post-translational alteration(s) of LPL is/are responsible for the increase in C-LPL activity. LPL is synthesized as an inactive monomer; acquisition of catalytic activity is the consequence of processing the glycoprotein in the endoplasmic reticulum and

Figure 1 Concentration response for insulin and dexamethasone in the stimulation of heparin-releasable LPL activity in cultured cardiomyocytes

Rat cardiomyocytes were incubated overnight with (a) 100 nM insulin and the indicated concentrations of dexamethasone or (b) 100 nM dexamethasone and the indicated concentrations of insulin. Results are means $\pm$ S.E.M. of 3 separate experiments for each panel.

subsequent dimerization [1,18]. The specific activity of total C-LPL in control cultured cardiomyocytes (0.069 mU/ng LPL protein; Table 2) is much lower than the value of 0.46 mU/ng LPL protein obtained for pure milk LPL [14], indicating that a substantial pool of inactive enzyme mass must be present in control cardiomyocytes. The hormone treatment would, therefore, appear to increase the ratio of active (dimeric) to inactive (monomeric) LPL. Incubation with INS/DEX also had no effect on LPL mass in cell lysates after heparin treatment; consequently, the specific activity of residual cellular LPL was increased from 0.047 to 0.075 mU/ng LPL protein by INS/DEX. The LPL released by heparin, which is assumed to be bound to heparan sulphate proteoglycans on the cell surface [1,4], was exclusively in the active form (0.44 $\pm$ 0.05 and 0.52 $\pm$ 0.08 mU/ng LPL protein for control and INS/DEX treatment, respectively) (Table 2). Our results suggest, therefore, that the increase in C-LPL

Table 2 Effect of insulin and dexamethasone on cellular and heparin-releasable LPL mass and specific activity

Cardiomyocytes were cultured overnight in the absence (control) or in the presence of 100 nM insulin plus 100 nM dexamethasone (INS/DEX). LPL mass and specific activity were measured in homogenates from cells incubated for 30 min with or without heparin and in the incubation medium of the heparin-treated cells. LPL mass in the medium of cells incubated without heparin was undetectable. Results are the means $\pm$ S.E.M. of 3 separate experiments. *P* values were calculated by a Student's paired *t* test; ***P* < 0.01, **P* < 0.05.

Experimental conditions	LPL mass (ng/mg)		LPL specific activity (mU/ng)	
	Control	INS/DEX	Control	INS/DEX
Cellular				
(–) Heparin	27.7 $\pm$ 0.7	28.6 $\pm$ 4.1	0.069 $\pm$ 0.008	0.113 $\pm$ 0.009**
(+) Heparin	25.6 $\pm$ 3.4	28.5 $\pm$ 6.3	0.047 $\pm$ 0.005	0.075 $\pm$ 0.008*
Medium				
(+) Heparin	8.7 $\pm$ 1.8	12.9 $\pm$ 1.2	0.44 $\pm$ 0.05	0.52 $\pm$ 0.08

specific activity produced by INS/DEX leads to an increased amount of fully active HR-LPL released into the medium.

These results obtained for the stimulatory effect of INS/DEX on LPL activity in cultured cardiomyocytes can be contrasted with work on adipocytes. First, insulin has a direct stimulatory effect on LPL activity in adipocytes as a consequence of either transcriptional [19], post-transcriptional [20] or post-translational [21] mechanisms, whereas insulin alone had no effect on LPL activity in cardiomyocytes (Table 1) in the absence of dexamethasone. Secondly, the potentiation of insulin activation of LPL in adipocytes by glucocorticoids was the result of increased transcription and LPL synthesis [7,9], or a post-translational mechanism where LPL degradation was decreased by dexamethasone [8]. In contrast, INS/DEX increased LPL specific activity in cultured cardiomyocytes with no change in LPL mass (Table 2). Thus, hormonal effects of INS/DEX on LPL activity provide another example of a tissue-specific mechanism for the regulation of LPL activity.

The stimulation of LPL in cultured cardiomyocytes by INS/DEX was a slow process (16 h overnight incubation) compared with the rapid stimulatory effect observed 1 h after insulin administration to control or diabetic rats [5,6]. Indeed, there was

no change in LPL activity when control cardiomyocytes that had been cultured overnight were incubated with INS/DEX for 2 h (results not shown). The effect of INS/DEX reported here, therefore, does not mimic the activation of LPL elicited by insulin injection. This emphasizes the complexity of the regulation of myocardial LPL *in vivo*. The findings reported here illustrate how the hormonal regulation of LPL can be studied *in vitro* with cultured cardiomyocytes, where individual variables can be carefully controlled.

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