Biochem. J. (1992) 285, 693–696 (Printed in Great Britain)

RESEARCH COMMUNICATION

Activation of lipoprotein lipase in cardiac myocytes by glycosylation requires trimming of glucose residues in the endoplasmic reticulum

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Incubation of cycloheximide-treated cardiac myocytes results in a time-dependent increase in cellular and heparin-releasable lipoprotein lipase (LPL) activities. *N*-Methyldeoxynojirimycin (1 mM) and castanospermine (100 μg/ml), inhibitors of glucosidases in the endoplasmic reticulum (ER), prevented the increase in cellular LPL activity. The glucosidase inhibitors did not influence the synthesis or turnover of LPL protein. Therefore activation of LPL by glycosylation in cardiac myocytes requires the trimming of glucose residues in oligosaccharide chains by glucosidases of the ER.

INTRODUCTION

Lipoprotein lipase (LPL) is synthesized in parenchymal cells (e.g. adipocytes and cardiac myocytes) and is then secreted and translocated to functional binding sites on the surface of vascular endothelial cells [1]. The endothelium-bound enzyme catalyses the hydrolysis of the triacylglycerol component of circulating lipoproteins. LPL is a glycoprotein with a carbohydrate content of 8–12% [2,3]. The process of *N*-linked glycosylation consists of [4,5]: (i) the co-translational transfer of dolichol-linked oligosaccharide to the nascent polypeptide; (ii) removal of terminal glucose residues by glucosidases in the endoplasmic reticulum (ER); (iii) transfer of the high-mannose glycoprotein to the Golgi apparatus; (iv) trimming of mannose residues by mannosidases in the *cis*/medial cisternae; and (v) synthesis of complex oligosaccharide chains by glycosyltransferases in medial *trans*-Golgi. LPL contains two complex oligosaccharide chains in mouse and rat adipocytes [3,6,7]; a precursor–product relationship between high-mannose and complex forms was observed in pulse–chase experiments. Mature LPL from guinea pig [8] and chicken [9] adipocytes contained three oligosaccharide chains: two complex and one high-mannose.

Glycosylation of LPL is required for its catalytic activity [1]. Synthesis of inactive and non-secretable LPL was observed when *N*-linked glycosylation was prevented by tunicamycin [7,10,11], an antibiotic which blocks formation of dolichol-linked oligosaccharides [4], or by glucose deprivation [12]. Furthermore, site-directed mutagenesis of one of the glycosylation sites (Asn-43) in human LPL resulted in the synthesis of inactive enzyme in COS cells [13].

From the above studies it is not clear if addition of the oligosaccharide is sufficient for LPL catalytic activity, or if processing is required. Treatment of Ob 17 pre-adipocytes with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to block the energy-dependent transfer of proteins from the ER to the Golgi [4] resulted in the accumulation of catalytically inactive LPL in the ER [14]. In contrast, monensin-treated cells were characterized as having active LPL that was retained in the Golgi but not secreted [7,14,15]; monensin inhibits the passage of glycoproteins from medial- to *trans*-Golgi cisternae [4]. Therefore, it was concluded that activation of LPL by glycosylation in Ob 17 cells was associated with processing of oligosaccharide chains in *cis*/medial Golgi [14]. Results with adipose 3T3-F442A cells have suggested, however, that activation of LPL was associated with processing of oligosaccharide chains to complex forms in *trans*-Golgi [3].

The objective of this investigation was to determine the effects of glycoprotein-processing-enzyme inhibitors [4,5] that inhibit trimming of glucose residues in the ER by glucosidases [*N*-methyldeoxynojirimycin (M-dNJ) and castanospermine (CS)] and trimming of mannose residues by mannosidase I [1-deoxymannojirimycin (dMM)] in the *cis*-Golgi on LPL activity in cardiac myocytes [16] and on the heparin-induced release of LPL into the incubation medium [17].

EXPERIMENTAL

Materials

Cycloheximide (CHX) and Staph A (Protein A, insoluble) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glycoprotein-processing inhibitors (M-dNJ, CS and dMM) were purchased from Genzyme Corp. (Boston, MA, U.S.A.). Trans ^{35}S-label (L-[^{35}S]methionine, L-[^{35}S]cysteine) was obtained from ICN Biomedicals (Mississauga, Ontario, Canada). The purified bovine milk LPL and affinity-purified chicken anti-(bovine LPL) antibody has been described [6]. Rabbit anti-chicken IgG was purchased from Cappel Research Products (Organon Teknika, Toronto, Ontario, Canada).

Abbreviations used: LPL, lipoprotein lipase; ER, endoplasmic reticulum; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; M-dNJ, *N*-methyldeoxynojirimycin; CS, castanospermine; dMM, 1-deoxymannojirimycin; CHX, cycloheximide.

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Preparation and incubation of cardiac myocytes

Ca^{2+}-tolerant myocytes were isolated from male Sprague–Dawley rats (250–300 g) as described previously [16]. LPL in cardiac myocytes was depleted by the administration of CHX (2 mg/kg; administered intraperitoneally) to the rat 2.5 h before removal of the heart [17,18]. CHX (50 μM) was also included in the isolation solutions during the preparation procedures [17]. Myocytes were washed twice to remove CHX, and were finally resuspended in fresh Joklik minimal essential medium supplemented with 1.2 mM-$MgSO_4$, 1 mM-DL-carnitine, 1.5 mM-$CaCl_2$, and 1% (w/v) defatted albumin to a cell density of 0.4×10^6 cells/ml. Heparin (5 units/ml) and glycoprotein processing inhibitors (M-dNJ or CS) were added at zero time, and the myocyte suspension was incubated at 37 °C under an atmosphere of O_2/CO_2 (19:1). After the indicated times of incubation, a 1 ml sample was removed and centrifuged for 15 s at 15000 *g* in an Eppendorf centrifuge. The supernatant (post-heparin medium) was removed, frozen and stored at −80 °C until assayed for LPL activity. The corresponding cell pellet after centrifugation was also frozen. Cell pellets were homogenized by sonication into either 1 ml of 0.25 M-sucrose/1 mM-EDTA/1 mM-dithiothreitol/10 mM-Hepes, pH 7.5 [16], or 200 μl of 50 mM-NH_4OH/HCl/0.125% (v/v) Triton X-100, pH 8.1 [6].

Assay for LPL activity

LPL activity was measured with a sonicated [^{3}H]triolein substrate emulsion [16], and is expressed as nmol of oleate released/h per 10^6 cells. The standard assay conditions were 0.6 mM-tri[9,10-^{3}H]oleoylglycerol (1 mCi/mmol), 25 mM-Pipes, pH 7.5, 0.05% (w/v) albumin, 50 mM-$MgCl_2$, 2% (v/v) chicken serum as LPL activator, and appropriate quantities of either the post-heparin medium (100 μl) or cell homogenate (5–20 μl) in a total volume of 400 μl [16]. When LPL activity was measured in cell homogenates, heparin (2 units/ml) was also included in the assay. The release of radiolabelled oleate was measured by liquid–liquid extraction [16] after a 30 min incubation at 30 °C. All LPL assays were performed under conditions in which the reaction was linear with respect to time and protein.

Cell labelling and LPL immunoprecipitation

CHX-treated myocytes in Joklik medium (10^6 cells/ml) were washed twice and finally resuspended into a modified Krebs–Henseleit buffer consisting of 118 mM-NaCl, 5 mM-KCl, 1.5 mM-$CaCl_2$, 1.2 mM-$MgSO_4$, 1.2 mM-KH_2PO_4, 24 mM-Hepes, 10 mM-D-glucose, 1% (w/v) albumin, pH 7.4, and [^{35}S]methionine (0.33 mCi/ml). Cells were labelled during a 60 min incubation period at 37 °C under O_2/CO_2 (19:1), and then 1 ml aliquots were centrifuged for 10 s at 15000 *g*. The cell pellets were sonicated into lysis buffer (1 ml) containing 3% (v/v) Triton X-100, 0.3% (w/v) SDS, 0.1% (w/v) *N*-dodecanoylsarcosine, 1 mM-phenylmethanesulphonyl fluoride and 0.1 M-Tris/HCl, pH 7.5. After centrifugation of the cell lysate at 15000 *g* for 20 min, a portion of the clear supernatant was removed to determine trichloroacetic acid-precipitable radioactivity in total protein. The remainder of the supernatant (0.8 ml) was then used for immunoprecipitation, using an affinity-purified chicken anti-(bovine LPL) antibody [6,19]. After the addition of an excess of rabbit anti-chicken IgG, the immune complexes were then precipitated with Staph A and subjected to SDS/PAGE [10% (w/v) mini-gel]. Gels were fixed in 10% (v/v) acetic acid and 40% (v/v) methanol for 15 min, treated for 1 h with Enhance for fluorography, 10% (v/v) glycerol and finally dried at 80 °C for 1 h. Dried gels were then exposed (with intensifying screens) to Kodak X-Omat XAR film at −70 °C for 7–10 days. The amount of [^{35}S]methionine-labelled LPL in the appropriate region of the gel was determined by liquid scintillation spectrometry, and is expressed as d.p.m. or as a percentage of the total radioactivity present in trichloroacetic acid-precipitable protein. A second immunoprecipitation of radiolabelled cell lysates did not reveal any radiolabelled LPL, indicating that the first immunoprecipitation was complete.

The turnover of LPL was assessed in pulse–chase experiments. Following a 20 min incubation of CHX-treated cardiac myocytes in modified Krebs–Henseleit buffer with [^{35}S]methionine, an aliquot was removed, centrifuged, lysis buffer was added to the cell pellet and radioactivity in immunoprecipitable LPL and in total trichloroacetic acid-precipitable protein was determined. The remaining cells were collected by centrifugation, resuspended in Joklik medium (with the normal concentration of 15 mg of methionine/l) and incubated for the indicated chase times before LPL immunoprecipitation.

The incorporation of [^{35}S] methionine into immunoprecipitable LPL in pulse and pulse–chase experiments was determined in cell lysates of CHX-treated myocytes after incubation in the absence of heparin, so that newly synthesized LPL protein would not be released into the incubation medium [17].

RESULTS AND DISCUSSION

CHX treatment (2 mg/kg) of rats *in vivo* for 2.5 h and the inclusion of 50 μM-CHX in the isolation solutions during the preparation of cardiac myocytes produces a marked reduction in cellular LPL activity to approx. 10% of control values [17]. Incubation of CHX-treated myocytes in fresh medium without CHX then results in a progressive increase in cellular LPL activity and, if heparin is present, in an increase in the release of LPL into the incubation medium (heparin-releasable LPL activity). Therefore, LPL is synthesized, processed and activated by glycosylation, and translocated to the cell surface during this

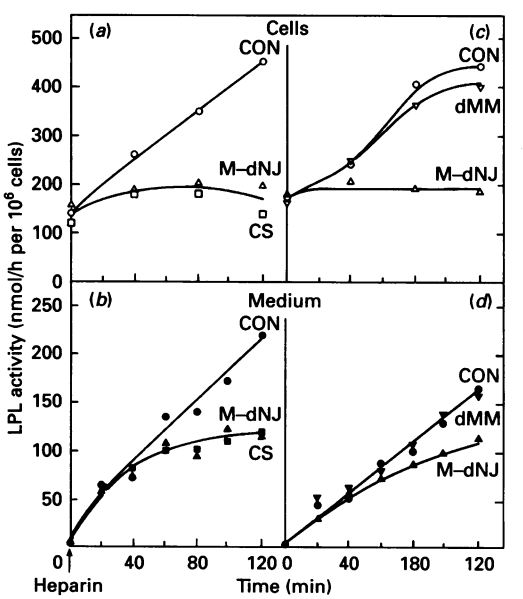

Fig. 1. Effect of glycoprotein-processing-enzyme inhibitors on cellular and heparin-releasable LPL activities in cardiac myocytes

CHX-treated cardiac myocytes were incubated with no additions (○, ●), 1 mM-M-dNJ (△, ▲), 100 μg CS/ml (□, ■), or 1 mM-dMM (▽, ▼). Heparin (5 units/ml) was added at zero time (arrow). After the indicated times of incubation, LPL activity was determined in homogenates of the cell pellets (open symbols) and in the post-heparin medium (closed symbols). CON, control.

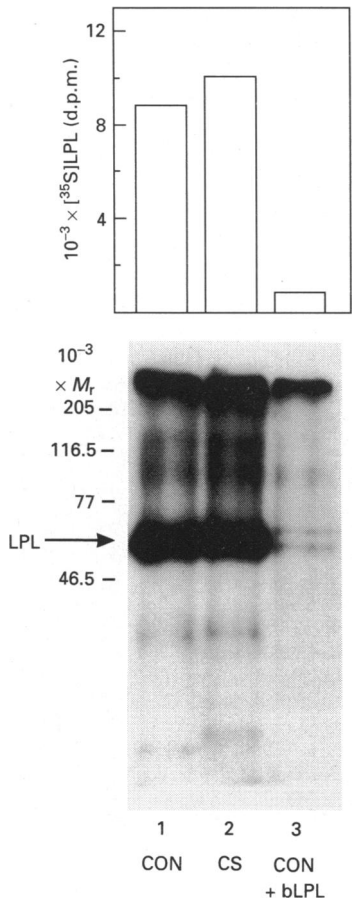

Fig. 2. Effect of CS on the synthesis of LPL

The radioactivity in immunoprecipitable LPL was measured (d.p.m.) after incubation of CHX-treated cardiac myocytes for 60 min with [^{35}S]methionine and either no other additions (CON, lane 1) or with 100 μg CS/ml (lane 2). In lane 3, 10 μg of purified bovine milk LPL (bLPL) was added together with the antibody before immunoprecipitation. The position of LPL in the autoradiogram is also shown (arrow), along with the position of M_r marker proteins. Similar results were obtained in a second experiment, where LPL radioactivity as a percentage of trichloroacetic acid-precipitable radioactivity in total protein was 0.056% and 0.052% for incubations in the absence and in the presence of CS respectively.

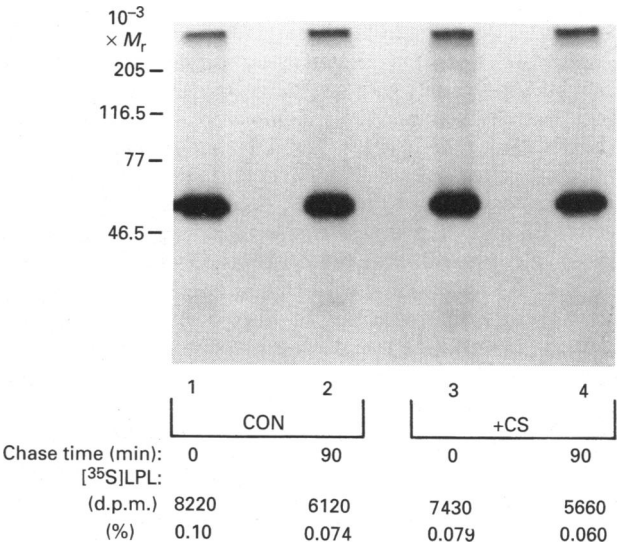

Fig. 3. Effect of CS on the synthesis and turnover of LPL

CHX-treated cardiac myocytes were pulse-labelled by a 20 min incubation with [^{35}S]methionine and either no further additions (CON, lane 1) or 100 μg of CS/ml (lane 3), followed by a 90 min chase incubation in the absence and in the presence of CS in Joklik medium (lanes 2 and 4 respectively). Following immunoprecipitation, the position of LPL on the gel was identified by comparison with the autoradiogram (position of M_r marker proteins shown). The content of [^{35}S]LPL, determined by liquid scintillation spectrometry, is expressed either as d.p.m. or as a percentage (%) of trichloroacetic acid-precipitable radioactivity in total protein. Similar results were obtained in two additional experiments with chase times of either 60 min or 120 min.

time interval. The increase in cellular and heparin-releasable LPL activities during a 120 min-long incubation of CHX-treated myocytes (CON) is shown in Fig. 1. It should be noted that the repletion of cellular LPL activity after this incubation (to 450 nmol/h per 10^6 cells) was incomplete; LPL activity in untreated cardiac myocytes ranges from 1400–2200 nmol/h per 10^6 cells.

The contribution of early steps in glycoprotein processing, that occur in the ER and *cis*-Golgi, to the activation of LPL was examined by using selective inhibitors of processing enzymes. M-dNJ (1 mM) and CS (100 μg/ml), inhibitors of ER glucosidases [4,5], blocked the increase in cellular LPL activity observed in CHX-treated myocytes (Figs. 1*a*, 1*c*) and reduced the heparin-induced release of LPL activity into the medium (Figs. 1*b*, 1*d*). The inhibitory effect of CS on repletion of cellular LPL activity was concentration-dependent, with 50% inhibition observed at a concentration of approx. 20 μg/ml. After a 2 h incubation of CHX-treated myocytes with M-dNJ and CS, the increment in cellular LPL activity was reduced to $15 \pm 3\%$ (mean $\pm$ S.E.M.; $n = 7$) and $15 \pm 6\%$ ($n = 5$) of control (no additions) respectively. The extent of the inhibition of heparin-releasable LPL activity by M-dNJ and CS was less than the reduction in the increment in cellular LPL activity, presumably because residual LPL activity (approx. 150 nmol/h per 10^6 cells; Fig. 1) in the CHX-treated cells would still be released in the presence of the processing-enzyme inhibitors. LPL activity in post-heparin medium was reduced by M-dNJ and CS to $60 \pm 3\%$ ($n = 7$) and $55 \pm 5\%$ ($n = 4$) of control respectively.

The effects of CS on the synthesis of LPL protein was determined to rule out the possibility that the reduction in the repletion of cellular LPL activity observed in Fig. 1(*a*) was caused by inhibition of LPL synthesis. Immunoprecipitable LPL following a 60 min incubation of CHX-treated myocytes with [^{35}S]methionine was identified as a protein band with an apparent subunit M_r of approx. 56000 after PAGE and autoradiography (Fig. 2, lane 1). Similar results have been obtained in pulse experiments with adipocytes [3,6–8] and whole heart [6,20]. Addition of excess purified bovine milk LPL with the antibody before immunoprecipitation markedly reduced the content of radioactivity in the 56000-M_r band (Fig. 2, lane 3), confirming the identity of the radiolabelled 56000-M_r band as LPL. CS did not reduce the incorporation of [^{35}S]methionine into immunoprecipitable LPL (Fig. 2, lane 2). The glucosidase inhibitor also had no significant inhibitory effect on the synthesis of LPL determined after a 20 min pulse incubation (Fig. 3, cf. lanes 1 and 3), or on the turnover of newly synthesized LPL after a 90 min chase incubation (Fig. 3, lanes 2 and 4). The turnover of LPL in cardiac myocytes, where approx. 75% of the radioactivity in immunoprecipitable LPL after the pulse incubation is still present following a 90 min chase incubation, is much slower than in adipocytes incubated in the absence of heparin [3,6,21], but is similar to the results of pulse–chase experiments in perfused guinea pig hearts [22].

While these studies on cardiac myocytes were in progress, Ben-Zeev *et al.* [23] reported that a 4 h incubation of CHO cells with CS and M-dNJ produced a concentration-dependent reduction in cellular LPL activity with no change in LPL mass, so that LPL specific activity was markedly diminished. The relatively slow rates of LPL synthesis (Figs. 1 and 2) and turnover (Fig. 3) in cardiac myocytes would make it difficult to determine accurately changes in enzyme mass; however, the absence of an inhibitory effect of CS on LPL synthesis and turnover in cardiac myocytes (Fig. 2) is consistent with the observation of inactive enzyme mass in CHO cells treated with glucosidase inhibitors [23]. It is clear, therefore, that trimming of glucose residues in the oligosaccharide chains of LPL in cardiac myocytes (Fig. 1) and CHO cells [23] by ER glucosidases is required for enzyme activity. Catalytically active LPL is a homodimer [1] and most proteins are oligomerized in the ER [24]. Therefore, trimming of glucose residues in the ER may produce conformational changes that induce dimerization and activation of LPL.

In contrast to these results with glucosidase inhibitors, inhibition of mannosidase I in *cis*-Golgi with dMM (1 mM) had no significant inhibitory effect on either cellular (Fig. 1*c*) or heparin-releasable (Fig. 1*d*) LPL activities in CHX-treated myocytes. Higher concentrations of dMM (4 mM) reduced the viability of myocytes. Treatment of adipocytes [7,8] and CHO cells [23] with dMM has been observed previously to result in a catalytically active form of LPL that was secreted. Therefore, Golgi processing is not required for the acquisition of catalytic activity by LPL. Semb & Olivecrona [8] isolated LPL from guinea pig adipocytes in a totally high-mannose form (all three chains) which was catalytically active. Furthermore, Ben-Zeev *et al.* [23] have shown convincingly that LPL is retained in the ER in a high-mannose, active form in CHO cells incubated at 16 °C to inhibit the vesicular transfer of glycoproteins from the ER to Golgi and in COS cells transfected with an LPL cDNA construct containing a specific tetrapeptide (KDEL) at the *C*-terminus as an ER retention signal. Vannier *et al.* [14] concluded that LPL activation was an event which occurred in the Golgi since treatment of Ob 17 pre-adipocytes with CCCP, to block the energy-dependent transfer of vesicles from the ER to the Golgi, resulted in the accumulation of inactive LPL in the ER. However, incubation of thyroid slices with CCCP decreased formation of dolichol-linked oligosaccharides and reduced protein glycosylation [25], therefore the inactive LPL in CCCP-treated Ob 17 cells may not have been glycosylated.

Similar results from experiments with glycoprotein-processing-enzyme inhibitors have been reported for hepatic lipase, another glycosylated lipolytic enzyme [26] which is a member of a lipase gene family which includes LPL [27]. M-dNJ and CS reduced intracellular hepatic lipase activity and inhibited the secretion of the enzyme from rat hepatocytes [28].

Received 19 May 1992; accepted 2 June 1992

This work was supported by an operating grant from the Medical Research Council of Canada and the National Institutes of Health (HL-28481, HL-21006) and the Veterans Administration.

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