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Inactivation of lipoprotein lipase in 3T3-L1 adipocytes by angiopoietin-like protein 4 requires that both proteins have reached the cell surface

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ABSTRACT

Lipoprotein lipase (LPL) and angiopoietin-like protein 4 (Angptl4) were studied in 3T3-L1 adipocytes. Transfections of the adipocytes with Angptl4 esiRNA caused reduction of the expression of Angptl4 to about one fourth of that in cells treated with vehicle only. This resulted in higher levels of LPL activity both on cell surfaces (heparin-releasable) and in the medium, while LPL activity within the cells remained unaffected. This demonstrated that even though both proteins are made in the same cell, Angptl4 does not inactivate LPL during intracellular transport. Most of the Angptl4 protein was present as covalent dimers and tetramers on cell surfaces, while within the cells there were only monomers. LPL gradually lost activity when incubated in medium, but there was no marked difference between conditioned medium from normal cells (rich in Angptl4) and medium after knockdown of Angptl4. Hence Angptl4 did not markedly accelerate inactivation of LPL in the medium. Experiments with combinations of different cells and media indicated that inactivation of LPL occurred on the surfaces of cells producing Angptl4.

1. Introduction

Lipoprotein lipase (LPL) is secreted from adipocytes. The enzyme undergoes trans-endothelial transport to the luminal side of capillaries where lipoproteins from plasma are hydrolyzed [1]. The main regulation of LPL activity in adipose tissue involves post-translational control of the balance between active LPL dimers and inactive monomers mediated by angiopoietin-like protein 4 (Angptl4) [2–4]. Angptl4 is a secretory protein produced in a number of cell types including adipocytes [5]. Like LPL, Angptl4 binds to cell surfaces and components of the extracellular matrix and is also found in blood. Full-length Angptl4 tends to form disulfide-linked complexes (dimers, tetramers and even higher oligomers) and is also prone to cleavage by proprotein convertases into an N-terminal domain (N-Angptl4), with ability to inactivate LPL, and a C-terminal fibrinogen-like domain with other functions [5]. The expression of Angptl4 is strongly up-regulated on fasting through activation of nuclear receptors by fatty acids [5] and is down-regulated on feeding through the actions of insulin [6,7]. Interestingly, Angptl4 also stimulates intracellular lipolysis in a glucocorticoid receptor-dependent manner [8]. Thus Angptl4 is considered to be a master switch for control of lipid storage [5].

We recently showed that Angptl4 is present as monomers within THP-1 macrophages, while in addition to monomers, covalent dimers and tetramers were found on the cell surface and in the medium [9]. Our data indicated that in the macrophage system inactivation of LPL by Angptl4 occurred mainly on the cell surface and was tightly connected to formation of Angptl4 oligomers.

Here we have used 3T3-L1 adipocytes to study the mechanism for inactivation of LPL by Angptl4. In adipocytes there is substantial LPL activity in the cells [10]. This enabled studies of whether or not Angptl4 acts on LPL already during the intracellular transport of the two proteins. Another experimental advantage with the adipocytes was the possibility to modulate Angptl4 expression by RNAi to verify the involvement of Angptl4 in the control of LPL activity.

2. Materials and methods

For more details see Supplementary material.

2.2. Cell culture and knockdown of Angptl4 by RNAi

3T3-L1 fibroblasts were grown in DMEM with 10% heat inactivated FCS (HI-FCS) and were differentiated 2 days post-confluence. Adipocytes were detached by TrypLE™ Express and treatment with

Abbreviations: Angptl4, angiopoietin-like protein 4; BSA, bovine serum albumin; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; esiRNA, endoribonuclease-prepared siRNA pools comprised of a heterogeneous mixture of siRNAs that all target the same mRNA sequence; HI-FCS, heat-inactivated fetal calf serum; LPD, lipoprotein-deficient; LPL, lipoprotein lipase; MIX, 3-Isobutyl-1-methylxantine; PBS, 10 mM phosphate, 0.15 M NaCl, pH 7.4.

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RNAi was performed in suspension [11]. MISSION® esiRNA mouse Angptl4 or MISSION® siRNA Universal negative control (Sigma–Aldrich, St. Louis, MO) was used with DharmaFECT® Duo transfection reagent (Thermo Scientific, Waltham, MA).

2.2. Recovery of samples from cell experiments

For most experiments the adipocytes were pre-washed with heparin (100 IU/ml DMEM + 0.2% BSA, 20–30 min at 37 °C) to remove Angptl4 and LPL from the cell surfaces in order to allow studies of newly secreted proteins. The cells were washed several times with fresh medium without heparin and then incubated at 37 °C in medium with or without HI-FCS, as detailed in the legends to the figures. Before collection of the media the plates were cooled for 10 min at 4 °C. Then the cells were briefly washed once with cold (4 °C) DMEM with or without 0.2% BSA. Then heparin-containing medium (100 IU/ml DMEM ± 0.2% BSA) was added and the cells were placed in a refrigerator for 30 min. This medium was recovered for measurements of LPL and Angptl4 in the "heparin-releasable fraction", representing proteins located at the cell surface. The remaining cells were washed with PBS and then dissolved either in solubilization buffer (0.025 M NH_4OH, 1 mg/ml BSA, 5 IU/ml heparin, 1% Triton ×100, 0.1% SDS with protease inhibitors) for analysis of LPL activity, in 0.2 M NaOH for measurement of total protein [9], in Protein Loading Buffer (Fermentas) for SDS–PAGE, or in lysis buffer from GeneJET RNA purification kit (Fermentas) with β-mercaptoethanol for analysis of mRNA.

2.3. Analyses of stability of mouse LPL in conditioned media

For analyses of the stability of LPL in culture media, 3 h-conditioned medium from Angptl4 esiRNA-treated cells containing 10% of HI-FCS was mixed on ice with the same volume of 3 h-conditioned, serum-free medium from either Angptl4 esiRNA-treated or control cells, and the mixtures were then incubated under cell culture conditions (37 °C, 5% CO_2). Aliquots were taken every 10 min for immediate analyses of remaining LPL activity.

2.4. Statistics

Statistical analysis of the data was performed using unpaired Student's *t* test.

3. Results

3.1. Knockdown of Angptl4 increases LPL activity on the cell surface and in the medium but not within the cells

At 5 days after induction of differentiation, and 24 h after change of medium, about 20% of the total LPL activity was in the medium, 30% was in the heparin-releasable fraction and 50% remained with the cells. Earlier studies have shown that while 3T3-L1 adipocytes continuously produce and secrete active LPL there is also inactivation of LPL in the system [10]. To study if Angptl4 is involved in the inactivation, its expression was knocked down by treatment with RNAi. A 70–80% reduction of the Angptl4 mRNA was attained by specific esiRNA (Fig. 1A). There was no significant effect of non-specific siRNA (negative control) on Angptl4 mRNA. LPL mRNA was not significantly affected (Fig. 1A). The amounts of Angptl4 protein in the heparin-releasable fraction, and remaining with the cells, were strongly reduced by treatment with Angptl4 esiRNA, as evaluated by Western blots (Fig. 1B), while LPL activity was increased several-fold, both in the heparin-releasable fraction and in the medium (Fig. 1C). In contrast, LPL activity remaining with the cells after the heparin release did not change significantly (Fig. 1C). These results indicate that Angptl4 did not act on LPL until both proteins were accessible for release by heparin, i.e. had reached the cell surface.

After removal of LPL and Angptl4 from the cell surfaces by heparin, LPL activity rose rapidly in fresh medium, but then gradually leveled off and reached a steady state where loss of activity balanced the delivery of new active enzyme (Fig. 2A). This is in line with previously published results [10]. With cells transfected with Angptl4 esiRNA the pattern was different. LPL activity in the medium increased almost linearly with time and was significantly higher than in medium from control cells (Fig. 2A). These results show that inactivation of LPL was markedly slowed down after Angptl4 esiRNA-transfection.

Western blots on the heparin-releasable fraction and medium from control cells at 3 h demonstrated that the amounts of Angptl4 protein were increased about 5- and 13-fold, respectively, compared to the 1 h time point (Fig. 2B). The ratios remained similar at 5 h.

3.2. Serum-depletion causes low LPL activity in medium but not in cells while the amounts of Angptl4 oligomers are increased in the heparin-releasable fraction

To analyze Angptl4 and LPL by Western blot it was necessary to grow the cells in medium without FCS (Fig. 2B). This had profound effects on the LPL system. In incubations without FSC, LPL activity was almost completely lost from media of both control and Angptl4 esiRNA-transfected cells (Supplemental Fig. S1A), while LPL activity within the cells, and levels of LPL mRNA, were not significantly changed (Supplemental Fig. S1B and C). The levels of Angptl4 mRNA and of Angptl4 protein mass within the cells tended to be higher after serum depletion in both Angptl4 esiRNA-transfected and control cells (Supplemental Fig. S1C and D), and a lot of Angptl4 was found in the medium from serum-depleted cells (Supplemental Fig. S1D). Only the full sized subunit of LPL (60 kDa) was present in the heparin-releasable fraction (Supplemental Fig. S1E). The LPL protein in this fraction dropped in the absence of serum, but was higher with cells treated with Angptl4 esiRNA. Both full-length LPL and a 38 kDa fragment were detected in heparin-washed cells as well as in the medium (Supplemental Fig. S1E). The amounts of full-length LPL in the cells and in medium remained similar irrespectively of treatment. The amount of the 38 kDa fragment was lower in medium from esiRNA-transfected cells than from control cells.

In order to further explore the effects of serum starvation, cells were grown for 5 h in medium with heat-inactivated lipoprotein deficient FCS (HI-LPD-FCS) or in medium with 2% BSA (Fig 3). Western blots revealed that the amount of heparin-releasable Angptl4 was markedly increased with cells grown with LPD-FCS, with BSA or in DMEM without any proteins (Fig. 3A). Running the blot analysis under non-reducing conditions demonstrated that all forms of Angptl4 (monomers, dimers and tetramers) were increased (Fig. 3B). The amounts of Angptl4 remaining with the cells after heparin wash tended to be higher in the absence of lipoproteins, but the difference was less pronounced than for the heparin-releasable fraction (Fig. 3A). LPL activity was strongly reduced in medium with HI-LPD-FCS, and even further reduced with BSA as the only protein (Fig. 3C).

3.3. Inactivation of LPL by Angptl4 does not occur in the medium, but on the cell surfaces

To study where the inactivation of LPL by Angptl4 occurred, control cells and Angptl4 esiRNA-transfected cells were grown for 3 h in medium with or without serum. Four different types of media were collected on ice: with normal Angptl4 level and