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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

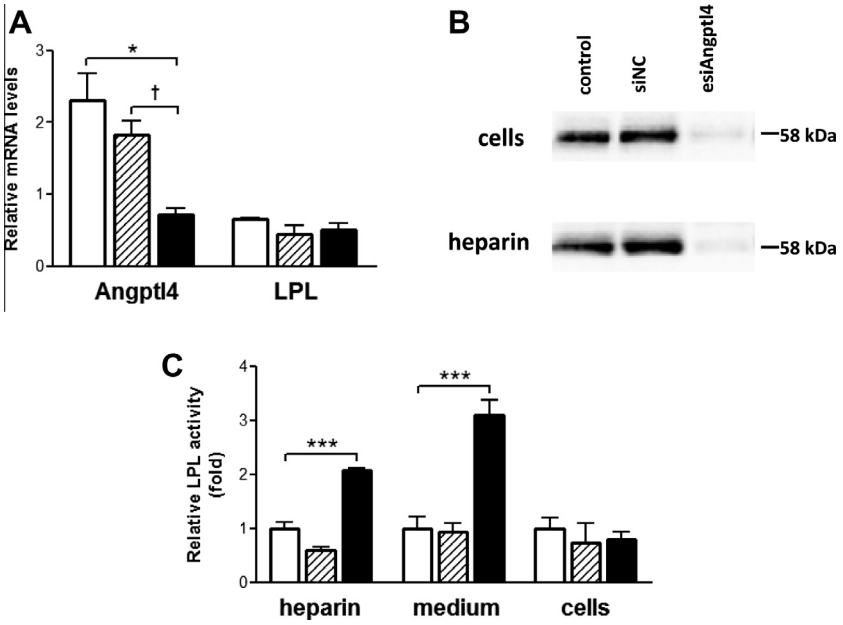

Fig. 1. Knockdown of Angptl4 affects LPL activity in 3T3-L1 adipocytes. Cells were treated with transfection reagent only (*open* bars, control), with non-specific siRNA (*hatched* bars, negative control) or Angptl4 esiRNA (*solid* bars) as described in Section 2 and recovered from transfection reagent for 72 h. (A) Angptl4 and LPL mRNA levels normalized to 18S rRNA; (B) Angptl4 protein in the cells and heparin-releasable fractions analyzed by Western blot (SDS–PAGE under reducing conditions). Samples applied to the lanes corresponded to 5 μg (*cells*) and 130 μg (*heparin*) of total cell protein and (C) LPL activity in heparin-releasable fraction (100 IU/ml, 30 min, 4 °C), 24 h-conditioned medium and in the cells, relative to control. The data are means of values from three wells ± SD $^{*}P < 0.05$ and $^{***}P < 0.001$ (Angptl4 esiRNA vs control); $^{\dagger}P < 0.05$ (Angptl4 esiRNA vs negative control siRNA).

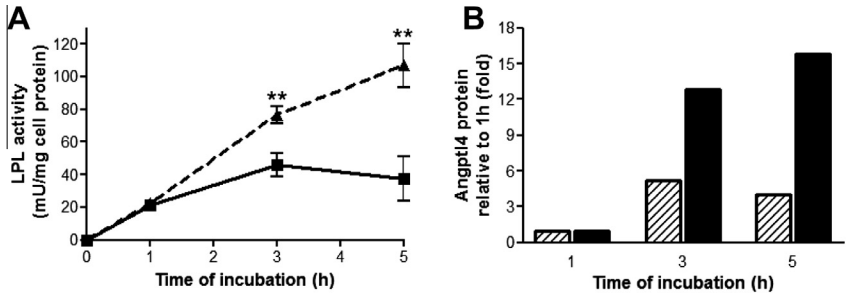

Fig. 2. Effect of knockdown of Angptl4 on build-up of LPL activity in the medium and Angptl4 protein in medium and on the cell surface. 3T3-L1 adipocytes were treated with transfection reagent only (control) or Angptl4 esiRNA and recovered from transfection reagent for 48 h. Then the surface-bound LPL and Angptl4 were removed with heparin (100 IU/ml, 20 min at 37 °C) and incubation continued in fresh medium with 10% HI-FCS for the indicated time. (A) Time courses for appearance of LPL activity in the medium from control (*solid* line) and Angptl4 esiRNA-transfected (*dashed* line) cells. The data are means of values from three wells ± SD $^{**}P < 0.01$ (Angptl4 esiRNA vs control) and (B) Analysis of Angptl4 protein on the cell surface (heparin release as detailed in Section 2, *hatched* bars) and in the medium (*solid* bars). The data represent scanning of Western blots (SDS–PAGE under reducing conditions) of samples corresponding to 120 μg of total cell protein.

normal LPL activity (from control cells grown with FCS), with low Angptl4 level and high LPL activity (from Angptl4 esiRNA-transfected cells grown with FCS), with high Angptl4 level and low LPL activity (from control cells grown without FCS) and with low Angptl4 level and low LPL activity (from Angptl4 esiRNA-transfected cells grown without FCS). Medium with high LPL activity was mixed with an equal volume of medium with high or low levels of Angptl4 and low LPL activity. The mixtures were incubated in wells without cells at 37 °C for 1 h and the decay of LPL activity was followed during this time (Fig. 4A). Despite the much higher amount of Angptl4 in the medium from control cells than from those transfected with Angptl4 esiRNA (see Fig. 4A insert), the time courses for inactivation of LPL were similar for both mixtures, indicating that the Angptl4 present in the conditioned medium was either not concentrated enough, or not in a form that could inactivate LPL.

In another set of experiments we used Angptl4 esiRNA-transfected adipocytes as source of LPL activity. After a heparin-wash cells were incubated in medium containing a final concentration of 5% HI-LPD-FCS with either freshly collected conditioned medium with high Angptl4 level and low LPL activity (see paragraph above) or fresh DMEM with purified recombinant mouse N-terminal Angptl4 (Fig. 4B). Mouse rN-Angptl4 at10 ng/ml caused a significant reduction in LPL activity in the medium already at 1 h, while 1 ng/ml was not sufficient. The build-up of LPL activity with the cells incubated with conditioned medium was the same as with 1 ng m-rN-Angptl4/ml (Fig. 4B). These experiments strongly indicated that the accelerated inactivation of LPL, caused by the presence of Angptl4 in the earlier experiments, did not occur in the medium. They also demonstrate that Angptl4 from medium could not efficiently bind in a proper way to the cell surfaces and cause inactivation of newly secreted LPL.

To directly study if the inactivation of LPL in conditioned medium required the presence of Angptl4-producing cells, Angptl4 esiRNA-transfected and control adipocytes were incubated in fresh medium with 10% HI-FCS (same conditions as in Fig. 2A) or in 24 h

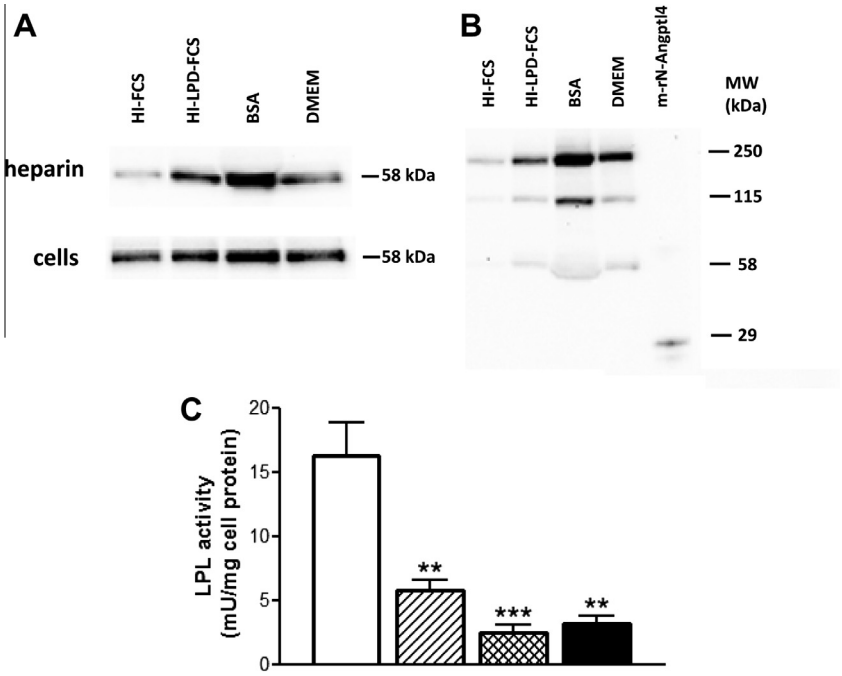

Fig. 3. Effect of lipoprotein depletion on LPL activity and Angptl4 protein. 3T3-L1 adipocytes were washed with heparin (100 IU/ml, 20 min at 37 °C) to remove surface-bound LPL and Angptl4. Then incubation continued for 5 h in fresh DMEM only, or in DMEM with either 10% HI-FCS, or 10% HI-LPD-FCS, or 2% BSA. (A) Angptl4 protein in the cells and heparin-releasable fractions analyzed by Western blot under reducing conditions. (B) Angptl4 protein in the cells and heparin-releasable fractions analyzed by Western blot under non-reducing conditions. Samples applied to the lanes corresponded to 10 μg (*cells*) and 100 μg (*heparin*) of total cell protein. Recombinant mouse N-Angptl4 (m-rN-Angptl4), 1 ng/lane, was used as control. (C) LPL activity in media. The data are means of values from three wells ± SD $^{**}P < 0.01$ and $^{***}P < 0.001$ (compared to medium with 10% HI-FCS).

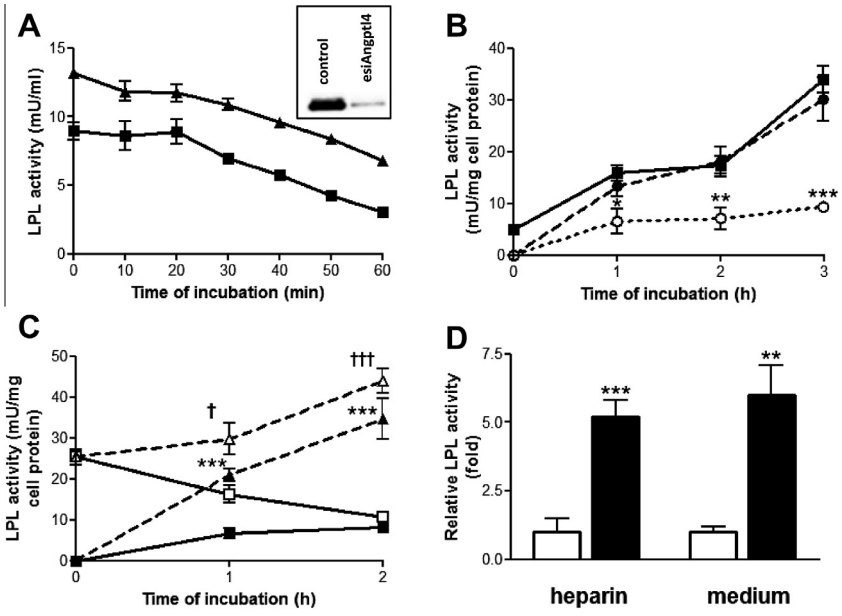

Fig. 4. The decisive factor for inactivation of LPL is the presence of Angptl4 producing cells. 3T3-L1 adipocytes were treated with transfection reagent only (control) or Angptl4 esiRNA and recovered for 48 h. Then the cells were washed with heparin (100 IU/ml, 20 min at 37 °C) and incubated in fresh medium with or without 10% HI-FCS for 3 h. Mixtures of these media were prepared as described in Section 2. (A) Inactivation of LPL at 37 °C in mixtures containing either conditioned medium from control cells (*squares*) or Angptl4 esiRNA-transfected cells (*triangles*). The *insert* demonstrates the difference in content of Angptl4 protein in the media without serum used for preparation of the mixtures (Western blot, reducing conditions); (B) LPL activity in the medium from Angptl4 esiRNA-transfected cells incubated either in mixtures of equal volumes of fresh medium with 10% HI-LPD-FCS and 3 h-conditioned serum-free medium from control cells (*solid* line), or in fresh medium with 5% HI-LPD-FCS containing mouse rN-Angptl4 (1 ng/ml (*long-dashed* line) or 10 ng/ml (*short-dashed* line)). The data are means of values from three wells ± SD $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (10 ng/ml rN-Angptl4 vs medium from control cells); (C) LPL activity in the medium from control cells (*solid* lines) or Angptl4 esiRNA-transfected cells (*dashed* lines) during incubation either in fresh medium (*solid* symbols) or in 24 h-conditioned medium from Angptl4 esiRNA-transfected cells (*open* symbols). The data are means of values from three wells ± SD $^{***}P < 0.001$ (Angptl4 esiRNA vs control in fresh medium), $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ (Angptl4 esiRNA vs control in 24 h-conditioned medium) and (D) Relation between LPL activity in the heparin-releasable fraction and in medium from control cells (set to 1, *open* bars) and from Angptl4 esiRNA-transfected cells (*solid* bars) after 3 h incubation in fresh medium. The data are means of values from three wells ± SD $^{**}P < 0.01$ and $^{***}P < 0.001$ (Angptl4 esiRNA vs control).

conditioned medium with high LPL activity (from Angptl4 esiRNA-transfected cells grown with HI-FCS). As expected, LPL activity produced by control cells into fresh medium reached a steady state after 1 h of incubation, while with Angptl4 esiRNA-transfected adipocytes the build-up of LPL activity in medium continued almost linearly (Fig. 4C).

LPL from Angptl4 esiRNA-transfected cells lost activity when added to control cells, but when added to esiRNA-transfected adipocytes the LPL activity instead increased with time reflecting secretion of new LPL from these cells. For cells incubated for 3 h in fresh medium, the LPL activity both in the heparin-releasable fraction and in medium from Angptl4 esiRNA-transfected cells was 5–6-fold higher than from the control cells (Fig. 4D). The fact that a similar fraction of LPL was active on the cell surfaces as in the medium indicated that the inactivation occurred already on the cell surfaces and that there was no further inactivation of LPL by Angptl4 in the medium from Angptl4-producing cells. This supports the view that inactivation of LPL by Angptl4 occurs on the plasma membrane of 3T3-L1 adipocytes, rather than in the medium.

4. Discussion

Angptl4 has emerged as an important regulator for LPL activity [3–5,9]. The lack of correlations between levels of Angptl4 in blood and plasma triglycerides and/or measures of LPL activity [7,12,13] indicate that Angptl4 may primarily act on LPL in adipose and other tissues where both proteins are produced. This arrangement appears appropriate, because LPL activity needs to be regulated in a tissue-specific manner [1]. Previous studies have demonstrated that covalent dimers, tetramers and higher oligomers of Angptl4 are needed for inactivation of LPL [14]. In a study with THP-1 macrophages we found that Angptl4 was present as monomers within the cells, but that dimers and tetramers were the dominating forms in the heparin-releasable fraction as well as in the culture medium [9]. That study indicated that inactivation of LPL occurred on the cell surfaces rather than in the culture medium [9].

Knockdown of Angptl4 in 3T3-L1 adipocytes led to increased LPL activity, both in the medium and in the heparin-releasable fraction. In contrast, there was no effect on LPL activity residing within the cells. This is in line with studies on modulation of LPL activity *in vivo*, demonstrating that extracellular LPL activity is much higher in adipose tissue of fed compared to fasted rats, but intracellular LPL activity is the same in the two states [15]. With Angptl4 esiRNA-transfected cells there was an almost linear build-up of LPL activity in the medium for several hours, demonstrating that LPL was rather stable and that the amounts of Angptl4 protein that were still produced, due to incomplete silencing, were not sufficient to inactivate LPL. The levels of full-length LPL protein were not affected by transfection with Angptl4 esi-RNA, neither in the cells nor in the medium, demonstrating that the inactivation was not due to fragmentation and/or degradation.

Both the total amount of Angptl4 and the proportion of dimers and tetramers were markedly increased in the heparin-releasable fraction when adipocytes were incubated in the absence of FCS. Under these conditions little or no LPL activity could be detected in the heparin-releasable fractions or in media, either from control cells or cells transfected with Angptl4 esiRNA, but there was no change of intracellular LPL activity compared to cells grown with FCS. The factor causing these dramatic changes was probably associated with lipoproteins, because incubation in DMEM with 10% HI-LPD-FCS, or DMEM with 2% BSA, had similar effects as incubation with DMEM without proteins. This effect of serum deficiency prevented detailed analyses of the possible correlation between LPL activity and amounts and oligomer forms of Angptl4 on the cell surfaces. The results indicated, however, that inactivation of LPL by Angptl4 at the cell surface could, under these conditions, be the mechanism for the almost complete loss of LPL activity.

It has been suggested that cleavage of Angptl4 by proprotein convertases is an important event for inactivation of LPL and effects on triglyceride metabolism, but as discussed by Mattijssen and Kersten the importance of the cleavage remains ambiguous [5]. We did not detect any fragmentation of Angptl4, although the antibody used was able to detect the recombinant N-terminal fragment of Angptl4. Thus, our data do not support the hypothesis that cleavage of Angptl4 is required for inactivation of LPL.

The half-life for LPL activity in conditioned medium did not differ much depending on whether the medium was from control cells or from cells transfected with Angptl4 esiRNA, and thus contained less of the Angptl4 protein. The half-life was in the frame of 40–60 min which is comparable to that previously reported for LPL activity in medium from 3T3-L1 adipocytes [10]. Addition of 10 ng m-rN-Angptl4/ml medium caused inactivation of LPL, while there was no effect of 1 ng/ml. Conditioned medium from control cells incubated without FCS was estimated (by Western blots) to contain about 20 ng Angptl4/ml, but this was not enough to cause inactivation of LPL. It is therefore possible that Angptl4 was secreted to the medium in a form that is not effective towards LPL. When medium containing active LPL was added to control cells with normal production of Angptl4, the LPL activity declined. In contrast, when added to Angptl4 esi-RNA transfected cells (with much lower production of Angptl4), the LPL activity increased at the same rate as when these cells were incubated in fresh medium, demonstrating that LPL inactivation required cells that were producing Angptl4.

We conclude that with 3T3-L1 adipocytes, inactivation of LPL by Angptl4 appears to occur after both proteins have traveled along the secretory pathway and arrived at the cell surface, as evidenced by being amenable to release by heparin. At or close to this site, monomers of Angptl4 form covalent dimers and tetramers similar to what we previously found using THP-1 macrophages [9]. It is likely that oligomerization of Angptl4 precedes inactivation of LPL. An interesting question is how this rapid self-association of Angptl4 is accomplished. If there are factors that prevent oligomerization within the cell and/or promote oligomerization on the plasma membrane they might have important regulatory effects on the LPL system, and maybe also on other functions of Angptl4.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.013.

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