Biochemical and Biophysical Research Communications 462 (2015) 33–37

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ShRNA-mediated gene silencing of lipoprotein lipase improves insulin sensitivity in L6 skeletal muscle cells

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ARTICLE INFO

Article history:
Received 15 April 2015
Available online 27 April 2015

Keywords:
Lipoprotein lipase
Glucose oxidation
Glycogen synthesis
Fatty acid oxidation
Insulin sensitivity
L6 skeletal muscle cells
shRNA

ABSTRACT

In previous studies, we demonstrated that down-regulation of lipoprotein lipase in L6 muscle cells increased insulin-stimulated glucose uptake. In the current study, we used RNA interference technology to silence the LPL gene in L6 cells and generate a LPL-knock-down (LPL-KD) cell line. ShRNA transfected cells showed a 88% reduction in the level of LPL expression. The metabolic response to insulin was compared in wild-type (WT) and LPL-KD cells. Insulin-stimulated glycogen synthesis and glucose oxidation were respectively, 2.4-fold and 2.6-fold greater in LPL-KD cells compared to WT cells. Oxidation of oleic acid was reduced by 50% in LPL-KD cells compared to WT cells even in the absence of insulin. The contribution of LPL in regulating fuel metabolism was confirmed by adding back purified LPL to the culture media of LPL-KD cells. The presence of 10 μg/mL LPL resulted in LPL-KD cells reverting back to lower glycogen synthesis and glucose oxidation and increased fatty acid oxidation. Thus, LPL depletion appeared to mimic the action of insulin. These finding suggests an inverse correlation between muscle LPL levels and insulin-stimulated fuel homeostasis.

1. Introduction

Lipoprotein lipase (LPL) is a lipolytic enzyme, required for the hydrolysis of triglycerides to glycerol and free fatty acids (FFA) [1,2]. LPL plays a key role in regulating the entry FFA into muscle and adipose cells, both insulin-responsive tissues [2,3]. LPL synthesis is regulated by various metabolic and endocrine stimuli in a tissue-specific manner [3], consistent with the different purposes of LPL activity in different tissues. In adipose tissue, LPL's function is to facilitate the entry of lipoprotein triglycerides into adipocytes for storage. Thus, activity of adipose LPL is increased in conditions of calorie excess. In the muscle, LPL activity releases FFA, which are oxidized for energy; thus, muscle LPL is induced during activity [3].

The skeletal muscle accounts for more than 75% of total insulin-stimulated glucose uptake. Accumulation of muscle fat is associated with reduced glucose utilization and insulin resistance. Since FFA contribute to insulin resistance, regulation of LPL expression and activity can effectively modulate insulin sensitivity. Muscle LPL levels appear to correlate with insulin resistance [4]. Previously, we have demonstrated that siRNA-mediated down-regulation of LPL in L6 muscle cells increases insulin-dependent glucose uptake into cells [5]. Down-regulation of muscle LPL may result in unavailability of FFA, which in turn, may force muscle cells to oxidize glucose for energy. Thus, LPL down-regulation appeared to mimic the action of insulin with regards to glucose uptake. Insulin is also known to increase glucose oxidation and glycogen synthesis, whereas it has an anti-lipolytic activity [6]. Thus the present study was to determine the effect of LPL down-regulation on glycogen synthesis, glucose oxidation and fatty acid oxidation in muscle cells. Our results demonstrate that silencing of muscle LPL improves glucose metabolism and insulin sensitivity.

2. Materials and methods

2.1. Cell culture and differentiation

Rat L6 skeletal muscle myoblasts were obtained from ATCC and maintained in growth media (DMEM supplemented with10% FBS (Atlanta Biologicals), 50units/mL penicillin, 50 μg/mL streptomycin, 10 mM HEPES, pH 7.4, and 2 mM glutamine) at 37 °C and 5% CO_2. Every 2–3 days the cells were 75–85% confluent and passaged at a sub-cultivation ratio of 1:10 using Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA). For experimentation, myoblasts were sub-cultured

Abbreviations: FFA, free fatty acids; LPL, lipoprotein lipase; LPL-KD, LPL knock-down; shRNA, short hairpin RNA; WT, wild-type.

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http://dx.doi.org/10.1016/j.bbrc.2015.04.098
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into 60 × 15 mm petri dishes in the presence of DMEM +10% FBS. By day 2, cells were 80–90% confluent and differentiated to myotubes by reducing the FBS content of the media to 2% for 2 days and subsequently to 0% for 2–4 days till several areas of fused myotubes were evident.

2.2. Silencing the LPL gene

RNA interference (RNAi) technology was used to generate a stable LPL knock-down (LPL-KD) L6 cell line. Lentivirus particles expressing rat LPL-specific short hairpin RNA (shRNA) were integrated into the host genome of L6 cells to silence the host's LPL gene. Cells were seeded into two T-25 cm^2 tissue culture flasks in growth medium at a density of 1×10^5 cells/mL, and incubated overnight at 37 °C and 5% CO_2. The next day, when cells were approximately 50% confluent, spent media was aspirated and replaced with 4 mL of a mixture of complete growth media supplemented with 5 μg/mL Polybrene. Cells were infected by adding 100 μL of LPL shRNA (rat) Lentiviral Particles (Santa Cruz Biotechnology, 0.5×10^4 infectious units/μL), chilled on ice for 15 min, and transferred to a 37 °C incubator for 48 h. The control flask was handled identically with the omission of Lentivirus. After 48 h, cells were washed, split from 1 to 3 flasks, and each flask was incubated in complete growth medium for 2 days. Cells stably transfected with the shLPL construct (designated as LPL-KD cells) were selected by treatment with 10 μg/mL puromycin dihydrochloride until all cells in the control flask were confirmed dead. The medium was replaced with fresh puromycin-containing medium every 2–3 days, until LPL silencing was confirmed by RT-PCR. For experiments, cells were differentiated to myotubes by culturing in serum-free medium for 4 days. Differentiation was confirmed by the presence of fused multinucleated long myotubes aligned lengthwise.

2.3. RNA isolation and RT-PCR

RNA was isolated from differentiated myotubes using TRI reagent (Sigma) and Direct-zol™ RNA miniprep kit (Zymo Research) according to the manufacturers' protocols. RNA was quantified by spectrophotometry at 260 nm and 4 μg of RNA was used to synthesize cDNA by reverse transcription using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), dNTPs, and oligodT primers (Promega).

End-point PCR was performed using cDNA and primer pairs shown (Table 1). The PCR amplicons were resolved by 2% agarose gel and the DNA bands were quantified by ImageJ (NIH) analysis. The cDNA was also subjected to real time quantitative PCR using a Smart Cycler (Cepheid Inc), RealMasterMix (5PRIME), and primer pairs shown (Table 1). A melting temperature (T_m) of 85 °C or higher was obtained, confirming primer-specific amplification. β-actin was used as the house-keeping gene control for both conventional and quantitative PCR. The threshold cycle (C_T) values were used to calculate fold change in transcript levels using the $2^{-\Delta\Delta C_T}$ method [7] as follows: Fold change $= 2^{-(C_{T\ \text{target}} - C_{T\ \beta\text{-actin}})_{\text{siRNA}} - (C_{T\ \text{target}} - C_{T\ \beta\text{-actin}})_{\text{control}}}$.

2.4. Metabolic studies in cell culture

To investigate the effects of LPL down regulation on insulin sensitivity, metabolic assays including glucose oxidation, glycogen synthesis, and fatty-acid oxidation were performed. WT and LPL-KD L6 cells were differentiated as described above. The differentiated myotubes (already serum-starved for differentiation) were simultaneously loaded with radiolabeled substrate (see below) and treated with or without Insulin (0.004 U/mL, Humulin R Lilly) for 2 h or 20 h prior to a metabolic assay. For 'LPL add-back' experiments, the cell culture medium was supplemented with 10 μg/mL of purified bovine LPL [8] during the incubation with insulin.

2.4.1. Glycogen synthesis assay

L6 myotubes in 12-well plates underwent 3 days of serum starvation for differentiation, following which they were incubated for 2 h or 20 h (20 h for LPL add-back experiments) in DMEM (4.5 mM glucose) without or with insulin (0.4 U/μL) in medium containing 0.15 μCi/mL of D-[U−^{14}C]glucose. Cells were then quickly washed in ice-cold PBS and lysed in 0.2 ml of 1 M KOH. Cell lysates were subjected to overnight glycogen precipitation with ethanol. Precipitated glycogen was dissolved in water and transferred to scintillation vials for counting radioactivity. Disintegrations per minute (DPM) were converted to moles of glucose using the specific activity of D-[U−^{14}C]glucose (300 Ci/mol) [9].

2.4.2. Glucose oxidation assay

Following 3 days of serum starvation, L6 myotubes in 60 × 15 mm Petri dishes were incubated with DMEM (1 mM glucose) medium containing for 0.15 μCi/mL of D-[U−^{14}C]glucose with or without Insulin (0.4 U/μL) for 2 h or 20 h (20 h for LPL add-back experiments). Each Petri dish was sealed with parafilm after a piece of Whatman paper was attached to the inside of the lid. The Whatman paper was wet with 100 μL of phenylethylamine-methanol (1:1) to trap CO_2 produced during the incubation period. After incubation, 200 μL of 4 M H_2SO_4 was added to the plates, followed by further incubation for 1 h at 37 °C. Finally, the Whatman paper were removed and transferred to scintillation vials for counting radioactivity [10].

2.4.3. Fatty acid oxidation assay

The procedure for measuring fatty acid oxidation was similar to that for glucose oxidation [11], except that 0.15 μCi/mL of D-[1−$_{14}$C] oleic acid was used instead of D-[U−$_{14}$C]glucose and for all experiments incubations with radiolabel and insulin were done for 20 h.

3. Results

In order to study the role of LPL in metabolic functions of insulin, we used shRNA lentivirus to silence the LPL gene in rat L6 skeletal muscle cells. Fig. 1 shows the complete absence of LPL mRNA in shRNA transfected L6 cells (designated as LPL-KD cells). Control cells were treated identically, but without the addition of shRNA lentivirus (WT), and showed abundant LPL message. There was no

Table 1
Primer sequences for end-point and real-time PCR.

Gene & amplicon Size (bp)	Primer type	Primer sequence
LPL (308 bp) (for end-point PCR)	Sense	5′-GGAATGTATGAGAGTTGGGT-3′
	Antisense	5′-GGGCTTCTGCATACTCAAAG-3′
LPL (139 bp) (for real-time PCR)	Sense	5′-CCCTAAGGACCCCTGAAGAC-3′
	Antisense	5′-TACATTCCTGTCACCGTCCA-3′
β-actin (285 bp) (for both end-point and real-time PCR)	Sense	5′-TCATGAAGTGTGACGTTGACATCCGT-3′
	Antisense	5′-CTTAGAAGCATTTGCGGTGCACGATG-3′

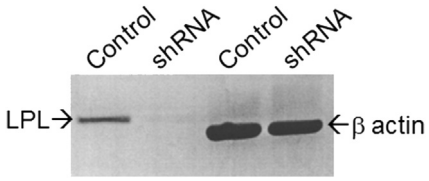

Fig. 1. Knock-down of *LPL* gene expression in L6 muscle cells using shRNA Lentivirus particles. Lentiviral Particles (Santacruz Biotechnology) were used to deliver a plasmid encoding LPL-specific shRNA to L6 myoblasts. Cells with stable expression of shRNA were selected by puromycin treatment and designated as LPL-KD L6 cells. Both WT and LPL-KD myoblasts were differentiated to myotubes as described. End-point RT-PCR analysis shows successful knock-down of LPL mRNA levels by 90% in shRNA transduced cells (shRNA) compared to wild-type (control) cells. β-actin was amplified as a control for shRNA specificity, RNA quantification, and gel loading.

difference in the expression of mRNA for β-actin, demonstrating that the shRNA specifically targeted only the LPL message. The β-actin PCR also served as a control for RNA mass and gel loading.

The absence of LPL in LPL-KD cells was also confirmed by real-time quantitative PCR. Table 2 shows the C_T values obtained for LPL and β-actin using a Cepheid SmartCycler. While the C_T values for β-actin were relatively similar in WT and LPL-KD cells, the C_T value for LPL was significantly higher in LPL-KD cells. Identical results were obtained after repeated experiments. The fold change was calculated using the using the $2^{-\Delta\Delta C_T}$ method [7] as follows: Fold change $= 2^{-(C_{T\ \text{target}} - C_{T\ \beta\text{-actin}})\text{siRNA} - (C_{T\ \text{target}} - C_{T\ \beta\text{-actin}})\text{control}}$.

This represented a 88% lower LPL message in shRNA transfected cells (LPL-KD) than in control (WT) cells.

One of the functions of insulin is to increase glucose utilization (glycolysis) and storage (glycogen synthesis) in muscle cells. We compared the insulin sensitivity of WT and LPL-KO cells by measuring the incorporation of ^{14}C-glucose into glycogen, and its oxidation to CO_2. Fig. 2A demonstrates the modulation of glycogen synthesis by LPL. For this experiment, cells were grown in complete growth media supplemented with excess (4.5 mM) glucose. In the presence of excess glucose insulin should promote glycogen synthesis. When cells were incubated in the absence of insulin, there is a slight increase in glycogen synthesis in LPL-KO cells compared to WT cells. The presence of insulin marginally increases glycogen synthesis in WT cells but dramatically induces glycogen synthesis in LPL-KO cells to more than twice that in the absence of insulin. Thus, depleting LPL from skeletal muscle cells sensitizes them to insulin.

The negative relationship between LPL protein and insulin sensitivity was confirmed by 'LPL add-back' experiments. Since LPL is a secreted protein, purified LPL protein was added exogenously to culture media of muscle cells simultaneously with insulin and the radioisotope. As shown in Fig. 2B, incubation of LPL-KO cells with 10 μg/mL purified bovine LPL reduced their ability to synthesize glycogen to a third of the level in the absence of LPL. Stimulation with insulin failed to enhance glycogen synthesis in these cells when LPL was present in the culture medium. As in Fig. 2A, insulin stimulated glycogen synthesis in LPL-KO cells in the absence of exogenous LPL. Thus, the presence of LPL brings about a resistance to insulin in these skeletal muscle cells.

To measure oxidation of glucose in muscle cells, their supply of glucose was restricted to 1 mM. This limited glycogen synthesis, instead routing the available glucose for harvesting energy via oxidation to CO_2. Stimulation with insulin increases glucose oxidation, as confirmed by the results shown in Fig. 3A. Insulin induced glucose oxidation in both WT and LPL-KO cells, by 64% and 84%, respectively. Interestingly, LPL silencing resulted in a 2.3 fold increase in glucose oxidation even in the absence of insulin stimulation. Thus, depletion of LPL mimicked the effect of insulin stimulation in muscle cells. Stimulation of LPL-KO cells with insulin further enhanced glucose oxidation to a level more than 4-fold higher than that in WT unstimulated cells.

The ability of LPL to suppress glucose oxidation was also demonstrated by replenishing LPL in LPL-KO cells by the addition of purified LPL to the culture media. The pattern of glucose oxidation in these cells reverted to that of WT cells. Glucose oxidation was lower in the presence of LPL and higher in the absence of LPL. Thus LPL-depletion appeared to have insulin-mimetic properties with the depletion of LPL and stimulation with insulin having synergistic effects.

Insulin has an anti-lipolytic effect. Thus insulin is expected to reduce fatty acid oxidation [6]. Depletion of LPL from muscle cells has a similar effect. As seen in Fig. 4A, LPL-KO cells exhibited only 50% of the oxidation of oleic acid seen in WT cells expressing LPL. Stimulation with insulin lowered fatty acid oxidation in WT cells, but in unstimulated LPL-KO cells, the oxidation of oleic acid was already below the level of insulin-stimulated WT cells, thus insulin did not significantly lower fatty acid oxidation in LPL-KO cells.

As expected, adding back LPL to LPL-KO cells increased fatty acid oxidation both in the presence and absence of insulin. The increase in the absence of insulin was 43% whereas in the presence of insulin the increase due to LPL addition was only 15%. In this assay also, it was evident that cells depleted of LPL behave as though they have been stimulated with insulin. Thus, silencing the LPL gene 'insulin-sensitizes' the cells.

Table 2
Real-time PCR C_T values and Quantification of LPL silencing in LPL-KD L6 cells. The level of LPL expression in LPL-KD cells relative to WT cells is shown in bold.

	Wild-type			LPL-KD		
	Replicates	Average	Std dev	Replicates	Average	Std dev
β-actin	17.46	17.39	0.39	20.29	19.65	0.77
	17.74			18.8		
	16.96			19.86		
LPL	28.82	29.07	0.21	34.09	34.47	0.60
	29.21			35.16		
	29.17			34.16		
Fold change		1.01	0.16		**0.12**	**0.04**

4. Discussion

In previous studies, we have shown that activation of PPAR-γ by Ciglitazone treatment of L6 muscle cells resulted in a down-regulation of LPL transcription and translation [5]. Thus, the thiazolidinedione-mediated improvement of muscle insulin sensitivity was shown to be mediated by LPL repression. Concomitantly, when LPL specific siRNA was used to silence LPL expression in rat skeletal muscle cells, there was a co-incident increase in glucose uptake. Thus, in this study, we investigated the wider scope of LPL's correlation to the metabolic actions of insulin. A role of LPL in regulating insulin action was demonstrated by two strategies, LPL deficiency and LPL abundance. A rat skeletal muscle cell line was made LPL-deficient by transducing a lentivirus expressing shRNA specific to the LPL gene. End-point PCR and real-time quantitative PCR demonstrated a 88% knock-down of the LPL gene in shRNA transduced (LPL-KO) cells. In another approach, the LPL-KO cells' culture media was replenished with excess purified bovine LPL. Since LPL is a secreted protein, this strategy was akin to LPL over-expression.

Our results clearly establish an inverse relationship between LPL levels and the metabolic actions on insulin. Glycogen synthesis and glucose oxidation in unstimulated LPL-KO cells was equal to or greater than the level in insulin-stimulated wild-type cells. LPL-KO cells stimulated with insulin showed even greater metabolic

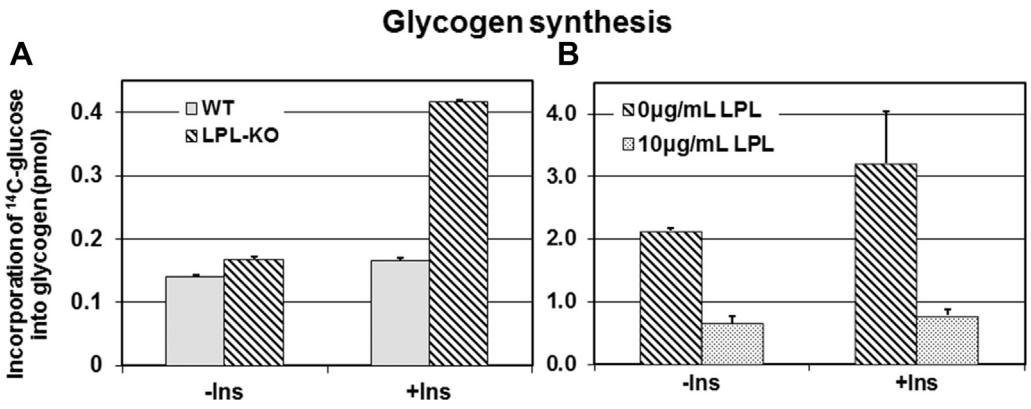

Fig. 2. Effect of LPL levels on glycogen synthesis. A. Serum-starved WT and LPL-KD myotubes were treated for 2 h without or with insulin (0.4 U/μL) in the presence of 0.15 μCi/mL of D-[U−^{14}C]glucose. The incorporation of labeled glucose into glycogen was measured as described in 'Methods'. B. Glycogen synthesis was measured as above in LPL-KD cells supplemented without or with 10 μg/mL purified LPL for 20 h simultaneously during incubation with the radioisotope. Results are averages of 3 replicates ± standard deviation.

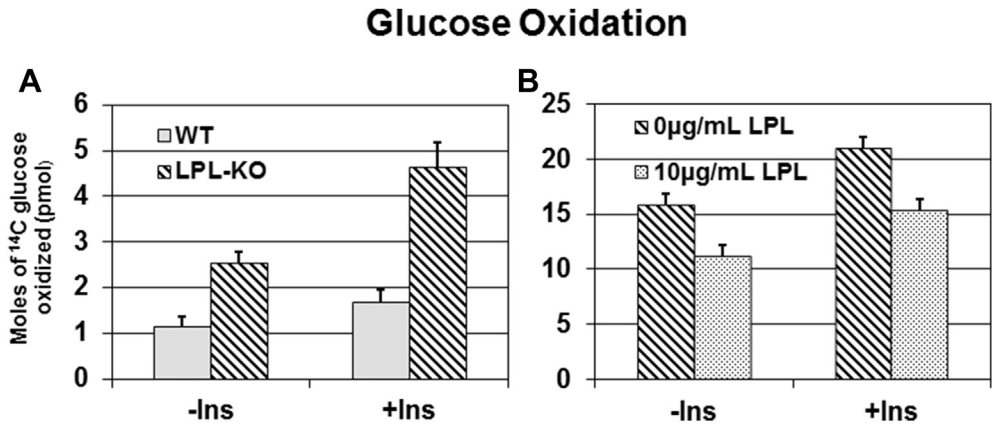

Fig. 3. Effect of LPL levels on glucose oxidation. A. Differentiated WT and LPL-KD myotubes in 60 mm dishes were incubated for 2 h in DMEM (1 mM glucose) containing 0.15 μCi/mL of D-[U−^{14}C]glucose and supplemented without or with insulin (0.4 U/μL). Glucose oxidation was measured as described in 'Methods'. B. Glucose oxidation was measured as above in LPL-KD cells supplemented without or with 10 μg/mL purified LPL for 20 h simultaneously during incubation with the radioisotope. Results are averages of 3 replicates ± standard deviation.

activity. On the contrary, silencing of the LPL gene reduces the oxidation of oleic acid mimicking the anti-lipolytic activity of insulin. Adding back LPL to the culture medium of LPL-KO cells reverses the metabolic patterns by reducing glycogen synthesis and glucose oxidation, but increasing fatty acid oxidation.

Our results are consistent with prior reports of studies in experimental animals [12–14]. Two studies with muscle-specific over-expression of LPL in transgenic mice showed impaired glucose tolerance and an increase in plasma glucose levels [12,13]. Kim et al. also showed a decrease in insulin-stimulated glucose

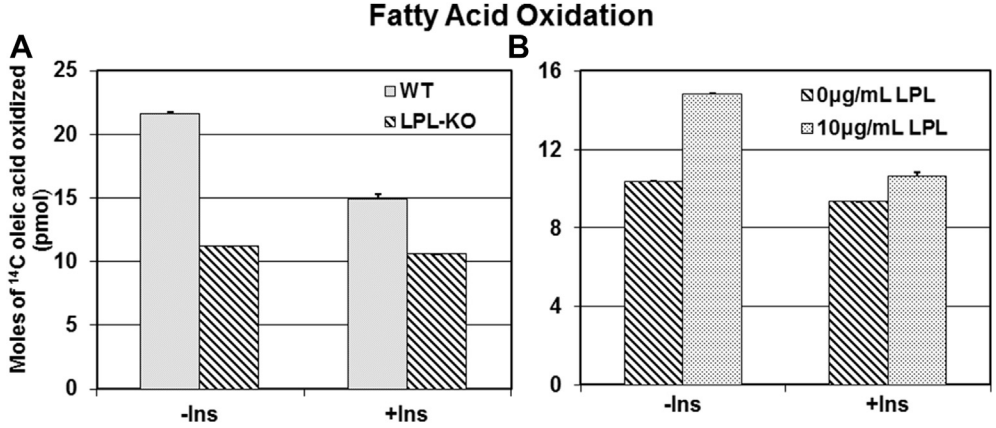

Fig. 4. Effect of LPL levels on oleic acid oxidation. A. Oxidation of ^{14}C-Oleic acid was measured in LPL-KD and WT L6 myotubes plated on 60 × 15 mm Petri dishes. Cells were incubated for 20 h in DMEM (1 mM glucose) containing 0.15 Ci/mL D-[1−^{14}C]oleic acid with or without insulin (0.4 U/μL). The amount of oxidized oleic acid was determined as described in 'Methods'. B. Oxidation of oleic acid was determined as above in LPL-KD L6 cells in the absence or presence of 10 mg/mL purified LPL added during the 20 h incubation with radiolabel. Results are averages of 3 replicates ± standard deviation.

uptake, glycolysis, and glycogen synthesis in the skeletal muscle of these mice [12]. In a reverse study, Eckel's group generated mice with skeletal muscle-specific LPL knockout (SMLPL−/−) [14], and consistent with our data, these mice showed increased insulin sensitivity. While the studies in whole animals are informative, cross-talk between different tissues hinder a clear understanding of the molecular events at the cellular level. Thus, our data obtained in cultured cells are significant, unambiguous evidence of the effect of skeletal muscle LPL on muscle metabolic activity.

There is a direct correlation between the level of free fatty acids (FFA) stored in the muscle and muscle insulin resistance [6]. LPL functions to make FFA available to the muscle cell for energy, and muscle-specific LPL over-expression correlated with accumulation of triglycerides in the skeletal muscle of transgenic mice [12]. Conversely, skeletal muscle-specific deletion of LPL resulted in a reduction in muscle triglyceride content [14]. In our study, LPL-KO muscle cells may improve their insulin sensitivity due to a repression of cellular FFA uptake. A depletion of available FFA in LPL-KO cells may force the uptake, oxidation and storage of glucose. The scarcity of fatty acids may also explain the decrease in fatty acid oxidation seen in the LPL-KO cells, consistent with insulin's anti-lipolytic activity.

LPL is not a traditional signaling molecule, however, ours and previous studies establish that modulation of LPL levels in skeletal muscle cells or tissue triggers events that affect the insulin signaling pathway. LPL overexpression in the skeletal muscle of transgenic mice correlated with a significant decrease in insulin-stimulated activation of insulin receptor substrate-1 (IRS-1) associated phosphatidylinositol 3-kinase activity (PI 3 Kinase). PI 3 Kinase is a key intermediate of the insulin signaling pathway, responsible for the metabolic functions of insulin in the muscle [12,15]. Mice with muscle-specific deletion of LPL showed an increase in insulin-stimulated phosphorylation of Akt, a downstream effector of PI 3 kinase; however the phosphorylation was independent of PI 3 Kinase activity, suggesting the involvement of another pathway [14]. Thus, in our studies, the down−regulation of LPL may trigger signaling events that may lead to metabolic perturbations described here. In future studies, it will be important to map the specific signaling pathway that connects the lack of skeletal muscle LPL to an 'insulin-stimulated' metabolic state.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by National Institutes of Health Awards R15HL083946 and SC3GM095413.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.098.

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