Increased expression of the SNARE accessory protein Munc18c in lipid-mediated insulin resistance

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Abstract   Fatty acids inhibit insulin-mediated glucose metabolism in skeletal muscle, an effect largely attributed to defects in insulin-mediated glucose transport. Insulin-resistant mice transgenic for the overexpression of lipoprotein lipase (LPL) in skeletal muscle were used to examine the molecular mechanism(s) in more detail. Using DNA gene chip array technology, and confirmation by RT-PCR and Western analysis, increases in the yeast Sec1p homolog Munc18c mRNA and protein were found in the gastrocnemius muscle of transgenic mice, but not other tissues. Munc18c has been previously demonstrated to impair insulin-mediated glucose transport in mammalian cells in vitro. Of interest, stably transfected C2C12 cells overexpressing LPL not only demonstrated increases in Munc18c mRNA and protein but also in transcription rates of the Munc18c gene. ⬛ To confirm the relevance of fatty acid metabolism and insulin resistance to the expression of Munc18c in vivo, a 2-fold increase in Munc18c protein was demonstrated in mice fed a high-fat diet for 4 weeks. Together, these data are the first to implicate in vivo increases in Munc18c as a potential contributing mechanism to fatty acid-induced insulin resistance.—Schlaepfer, I. R., L. K. Pulawa, L. D. M. C-B. Ferreira, D. E. James, W. H. Capell, and R. H. Eckel. **Increased expression of the SNARE accessory protein Munc18c in lipid-mediated insulin resistance.** *J. Lipid Res.* **2003.** 44: **1174–1181.**

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A recent report from the Centers for Disease Control and Prevention indicates that diabetes in the United States rose by about 6% in 1999, a trend the government called "dramatic evidence of an unfolding epidemic." The rise is blamed largely on obesity, which has increased by 57% since 1991 (1). A common feature of obesity and Type 2 diabetes is insulin resistance, which is highly associated with dyslipidemia, hypertension, and atherosclerosis. Availability of FFA is thought to play an important role in the development of insulin resistance (2).

Lipoprotein lipase (LPL) is pivotal in the trafficking and partitioning of lipoprotein-derived fatty acids and is most abundant in adipose tissue, heart, and skeletal muscle (3). To evaluate the importance of LPL on substrate partitioning in skeletal muscle, we developed a transgenic mouse with muscle-specific overexpression of human LPL (MCKhLPL). MCKhLPL mice were less susceptible to diet-induced obesity, and had lower plasma triglycerides and FFA, but were insulin resistant (4, 5).

Insulin resistance can be defined in part as a reduced capacity for insulin to increase glucose uptake and metabolism in target tissues such as skeletal muscle and adipose tissue. In normal subjects, insulin stimulates glucose transport primarily by inducing translocation of a facilitative glucose transporter isoform, GLUT4, from intracellular compartments to the plasma membrane (6). In recent years, the number of proteins implicated in GLUT4 vesicular transport has grown tremendously. Among them, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins play a key role in the docking and/or fusion between the transport vesicle (v-SNARE, e.g., VAMP2) and the target membrane (t-SNARE, e.g., syntaxin 4 and SNAP23) (7). Additionally, several other protein families regulate the interaction between the SNAREs and may either prevent or promote SNARE complex formation leading to membrane fusion.

Abbreviations: EST, expressed sequence tag; DGAT, diacylglycerol acyltransferase; PI 3-kinase, phosphatidylinositol 3-kinase; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

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Munc18 is a syntaxin binding protein and is the mammalian homolog of the yeast Sec1p and *Caenorhabditis elegans* Unc18 proteins. Presently, three mammalian Munc18 isoforms have been defined, Munc18a, -b, and -c (8). The third isoform, Munc18c, is a ubiquitous syntaxin 4 binding protein, which has been shown to specifically modulate insulin-sensitive GLUT4 translocation in fat and muscle in vitro. Overexpression of Munc18c in 3T3-L1 adipocytes and murine skeletal muscle inhibited insulin-stimulated glucose transport, whereas overexpression of the Munc18b isoform (which does not bind syntaxin 4) produced no effect (9). These studies imply a potential regulatory role of Munc18c in GLUT4 translocation and glucose transport. The defects in GLUT4 translocation that underlie insulin resistance in muscle are still obscure, but there is substantial evidence to support an inverse relationship between insulin sensitivity and lipid availability in muscle (10–12).

To gain further insight into an explanation for the LPL-mediated insulin resistance, we performed DNA microarray analysis using total RNA from gastrocnemius muscles of MCKhLPL and control littermate mice. In this report, we show that Munc18c is up-regulated in the muscle of two models of insulin resistance, MCKhLPL and high-fat-fed mice, and that this up-regulation also occurs in LPL-stably transfected myocytes. This is the first demonstration in vivo that increases in Munc18c in skeletal muscle may, in part, explain fatty acid-mediated insulin resistance.

MATERIALS AND METHODS

Animals

Mouse experiments were carried out according to approved protocols from the animal care committee at the University of Colorado Health Sciences Center (UCHSC). All mice (male) were housed at ~20°C on a 12:12 h light-dark photoperiod and had unrestricted access to water and food. For the DNA chip, RT-PCR, and protein analyses, MCKhLPL mice and control littermates on a FVB background were maintained on standard laboratory chow. Another set of nontransgenic FVB mice were fed either a synthetic high-fat diet (46% kcal, #D12344, Research Diets, New Brunswick, NJ) or a low-fat diet (11.5%, #D11724, Research Diets) for 4 to 5 weeks. All tissue extractions were performed on mice anesthetized with 250 mg/kg Avertin (2,2,2 tribromoethanol; Aldrich, Milwaukee, WI) after a 4 h fast. The gastrocnemius muscle was used for RNA extraction and protein analyses. Only MCKhLPL mice and their littermate controls on the chow diet were used for the Munc18c tissue survey analyses (adipose tissue, liver, brain, heart, spleen, and lung). Interperitoneal (ip) glucose tolerance tests were performed on nontransgenic FVB mice fed the high-fat and low-fat diets. Blood was sampled by tail bleed after a 4 h fast to measure basal blood glucose using a One Touch Profile blood glucose meter (Lifespan, Milpitas, CA). After the initial blood sampling, both groups of mice received a 1 g/kg of 20% dextrose injection administered ip. Blood glucose levels were monitored at 10, 20, 30, 60 and 120 min after dextrose injection.

Cell culture

C2C12 cells, stably transfected with human LPL (C2C12-LPL) and C2C12-control cells were plated and expanded (13). Cells used for protein and RNA extraction were harvested and stored at −80°C.

Total RNA isolation

Total RNA of muscle tissue and C2C12 cells and protein fractions of C2C12 cells were extracted with TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to manufacturer's protocol. Further purification of the total RNA was made with the RNeasy Mini kit from Qiagen (Valencia, CA).

Microarray analysis

Preparation of the labeled RNA from purified RNA of MCKhLPL and control littermate muscle samples for chip analysis was carried out following the protocol from the Affymetrix GeneChip expression analysis manual (Santa Clara, CA). The biotin-labeled antisense cRNA was produced using the ENZO Bioarray RNA transcript labeling kit (Farmingdale, NY). Biotin-labeled cRNA was purified by using an RNeasy affinity column (Qiagen) and fragmented, quantified, and hybridized to Mu11KsubA chips at the UCHSC microarray core. To examine the impact of LPL overexpression in the skeletal muscle gene profile of MCKhLPL mice, four GeneChip arrays from Affymetrix (two control and two MCKhLPL) were performed. The minimum acceptable change was set at 1.85-fold. The list of genes generated by the chips revealed a few known genes but mostly differentially expressed sequence tag (EST) clones in MCKhLPL versus control mice. EST clone sequences were matched to sequences of characterized genes via database homology searches using the National Center for Biotechnology Information server (www.ncbi.nlm.nih.gov/BLAST). Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been previously described (14).

RT-PCR

Three to five micrograms of the purified RNA was used for semiquantitative RT-PCR as described earlier (8). The following primers were used in the RT-PCR reactions of this study: Munc18c, 5′-TGATTCGTAACTGGAGCCAC-3′ (sense) and 5′-TTTCTGG-CGAGCACTCACA-3′ (antisense); GLUT4, 5′-TGGGATCCTGG-TATCTCAGC-3′ (sense) and 5′-TGATCAGCTTTTGTTCGTCG-3′ (antisense); syntaxin 4, 5′-CGAGTTCTTCCAGAAGGTGC-3′ (sense) and 5′-ACAAATTGCTGGGACAGGAC-3′ (antisense). Primers were designed with the help of Primer3 Input program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and made by Gibco BRL custom oligonucleotide service.

Western analysis

Tissue homogenates were prepared by homogenization in 1% Triton X-100 in Tris-buffered saline, pH 7.4 (20% w/v), and centrifugation at 12,000 *g* for 10 min. Protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) according to the manufacturer's instructions.

Protein samples (30 μg) in Laemmli sample buffer were boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were then transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked overnight with 5% nonfat dried milk in TBS [20 mM Tris-HCL (pH 7.4), 100 mM NaCl, and 0.05% Tween 20] and then incubated for 1 h with primary antibody diluted in TBS solution. After washing with TBS, membranes were incubated for 45 min with horseradish peroxide-conjugated antibodies to rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1:10,000 in TBS. After washing three times in TBS for 15 min, immune complexes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). Western blots were analyzed using either AlphaImager (Alpha Innotech Corp., San Leandro, CA) or SigmaGel (SPSS Science, Chicago, IL).

LPL-derived FFA from artificial triglyceride substrate by C2C12-LPL and C2C12-control cells

C2C12-LPL and C2C12-control cells were grown to confluency and then introduced to an experimental medium containing an artificial triglyceride substrate. The artificial triglyceride substrate was prepared by sonicating aliquots of Intalipid® 20% IV fat solution (Baxter, Deerfield, IL), 10% FFA-free bovine albumin solution (Sigma, St. Louis, MO), fasting, human serum to provide apolipoprotein C-II, and Kreb's Ringers phosphate buffer (pH 7.4). Triglyceride substrate was then combined with nonsupplemented, low-glucose DMEM with glucose added to create the experimental media with glucose concentration of 12 mM and triglyceride concentration of 200 mg/dl.

C2C12-LPL and C2C12-control cells were maintained in experimental media at 37°C in an atmosphere of 95% air-5% CO_2 and 100% humidity. Samples were taken from the media at 0, 4, 8 and 12 h. To assess the stability of the triglyceride substrate at 37°C, experimental media were plated without cells and sampled at the time points noted above. Media samples were immediately frozen and stored at −20°C until analyzed. Media FFAs were measured by enzymatic methods (Wako, Richmond, VA). FFA measurements were standardized by cell protein content determined using the BCA method.

Isolation of nuclei and nuclear run-on assay

C2C12 cells expressing LPL at high levels and low levels were grown to confluency and harvested for nuclei isolation as described in Current Protocols on Molecular Biology (15) with minor modifications. Briefly, cells were homogenized in Buffer I [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM Mg acetate, 0.1 mM EDTA, 10 mM Tris-HCL (pH 8.0), 1 mM DTT, and 0.5% NP-40] and mixed with 2 vol of 3 M sucrose in Buffer I. This mixture was then layered onto a cushion of 2.3 M sucrose in Buffer I and centrifuged at 30,000 g for 45 min at 4°C. The nuclear pellet was resuspended in glycerol storage buffer [50 mM Tris-Hcl (pH 8.3), 40% v/v glyc-

erol, 5 mM $MgCl_2$, and 0.1 mM EDTA) and stored in liquid nitrogen. Blots for the nuclear run-ons were prepared using equal amounts of linearized probes (5 µg) denatured with alkali and individually blotted onto positively charged nylon membranes. Nuclear run-on assays were carried out as described by Zhang et al. (16) using DIG-11-UTP as the labeling nucleotide instead of [α-^{32}P]UTP. Nuclei (5×10^7) were mixed with an equal volume of $2\times$ transcription buffer [10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.3 M KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.6 mM UTP, 0.4 mM DIG-11-UTP, and 5 mM DTT) and incubated at 30°C for 30 min with constant agitation. The reaction was terminated with the addition of 3 vol of TRIzol, and the RNA was then purified as indicated by the manufacturer. The purified RNA was heat denatured and added to the hybridization buffer (NorthernMax Hyb Buffer, Ambion, Austin, TX). Hybridization of DIG-labeled nascent nuclear RNAs to DNA slot-blots containing mouse Munc18c, human LPL, empty vector (negative control), and diacylglycerol acyltransferase (DGAT) genes was performed in 2 ml screw-cap tubes at 50°C overnight with rotation. Blots were washed twice in $2\times$ SSC-0.1% SDS at room temperature for 10 min and then at 68°C for 20 min in $0.1\times$ SSC-0.1% SDS. Chemiluminescent detection of the DIG-RNA hybridized to the blots was carried out using the DIG Luminescent Detection Kit from Roche Diagnostics (Mannheim, Germany). Blots were scanned using the AlphaImager, and the signals for Munc18c were normalized to the DGAT signal.

RESULTS

Gene array, RT-PCR, and Western analysis

To examine the impact of LPL overexpression on the skeletal muscle gene profile of MCKhLPL mice, Gene-Chip arrays from Affymetrix were performed. The list of genes generated by the microarray analysis included an
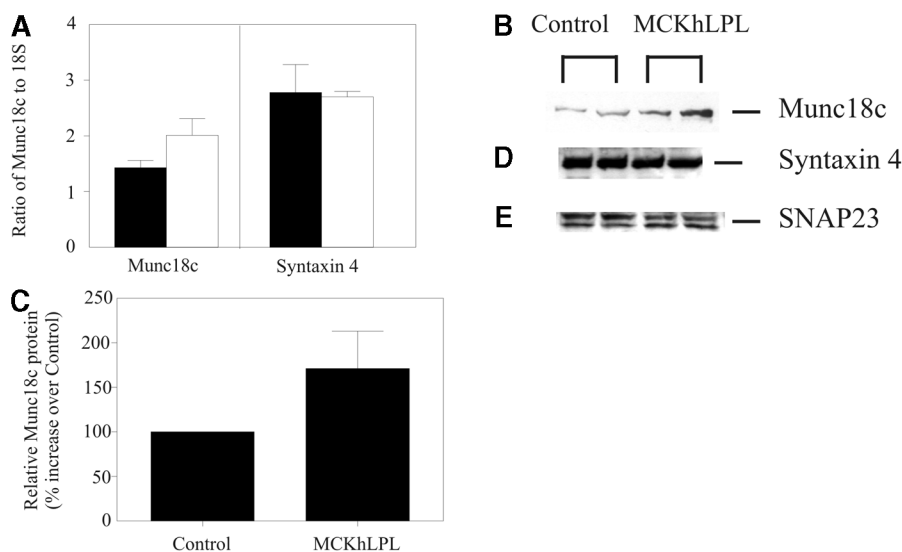

Fig. 1. Munc18c mRNA and protein levels in transgenic mice with muscle-specific overexpression of human lipoprotein lipase (MCKhLPL) muscle. Results are shown as mean ± SE. A: Cumulative results of RT-PCR for Munc18c and syntaxin 4 transcripts in gastrocnemius samples of MCKhLPL (n = 6) and control mice (n = 6). Closed and open bars represent control and MCKhLPL transcripts, respectively. The standard for RNA input in the RT-PCR analysis was 18S rRNA. B: A representative Western blot for Munc18c demonstrating the increased expression of Munc18c protein in gastrocnemius samples of MCKhLPL mice versus control littermates. C: Percent increase of Munc18c protein in the gastrocnemius of MCKhLPL mice (n = 7) relative to control (n = 6). Representative immunoblots of syntaxin 4 (D) and of SNAP23 (E) in the gastrocnemius of control and MCKhLPL mice.

EST clone with high homology to the Munc18 gene (17), which showed ~2-fold mRNA increase in MCKhLPL mice compared with control littermates.

To confirm the microarray results, RT-PCR primers to the Munc18c isoform (8) were synthesized and used for semiquantitative RT-PCR reactions as previously described (18) using the 18S rRNA as a standard. Most of the MCKhLPL muscle samples presented a 1.4- to 1.7-fold elevation of Munc18c mRNA compared with controls (**Fig. 1A**); however, the consistency of this moderate increase led us to investigate Munc18c protein content. Western analysis on MCKhLPL muscle homogenates also showed an increase in Munc18c protein level in MCKhLPL versus control mice that paralleled the RNA data (Fig. 1B, C). Syntaxin 4 mRNA (Fig. 1A) and protein levels (Fig. 1D) were not different between MCKhLPL and control mice. RT-PCR analysis for GLUT4 mRNA was not different between MCKhLPL and control mice (unpublished observations). There were also no changes in SNAP23 (Fig. 1E) or VAMP2 (unpublished observations) protein content in MCKhLPL muscle samples compared with control mice.

Munc18c up-regulation is specific to skeletal muscle of MCKhLPL mice

In view of the Munc18c results in the skeletal muscle of MCKhLPL mice, we then sought to determine whether overexpression of Munc18c occurs in other MCKhLPL tissues. The insulin-responsive tissues, adipose tissue (**Fig. 2A**), and liver (Fig. 2B), did not show increases in Munc18c. Brain, heart, spleen, and lung also failed to demonstrate a change in Munc18c levels (unpublished observations).

Up-regulation of Munc18c in high-fat-feeding-induced insulin resistance

To further study the link between lipid-mediated insulin resistance and Munc18c overexpression in muscle, we placed FVB mice on a low-fat or high-fat synthetic diet for at least 4 weeks. Rodents placed on a high-fat diet develop skeletal muscle insulin resistance within 3 to 4 weeks (19–21). For this study, ip glucose tolerance tests were performed after 4 weeks on the diet as an indicator of insulin resistance (**Table 1**). After sacrifice, the muscle samples were used for Munc18c Western analysis. The muscle samples from high-fat-fed mice showed a ~2-fold increase of
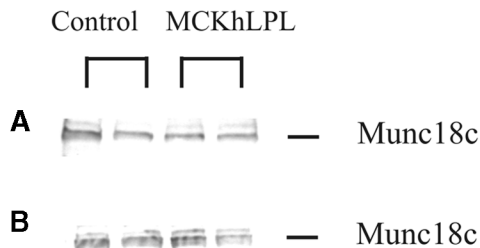
TABLE 1. Blood glucose levels in low-fat- and high-fat-fed mice after ip injection of 1 g/kg glucose

Time	LF	HF
min	*mg/dl*	
0	99 ± 17	162 ± 11
10	163 ± 26	251 ± 21[a]
20	193 ± 19	318 ± 30[a]
30	195 ± 19	348 ± 261[a]
60	171 ± 24	344 ± 26[a]
120	118 ± 12	287 ± 21[a]

LF, low fat; HF, high fat. Values are means ± SE. Repeated measures two-way ANOVA, LF versus HF, $P = 0.005$. Tukey pairwise comparison.
[a]$P < 0.05$ versus LF.

Munc18c protein when compared with muscle samples from the low-fat-fed mice (**Fig. 3**).

C2C12 cells transfected with LPL overexpress Munc18c and show an increase in LPL-derived FFA release

Our laboratory previously reported the generation of an in vitro model of LPL overexpression in C2C12 muscle cells (13). C2C12 cells stably transfected with human LPL cDNA (C2C12-LPL) showed a significant 2-fold increase in Munc18c mRNA when compared with the C2C12-control cells (**Fig. 4A**). Western analyses of C2C12-LPL and C2C12-control cells (Fig. 4B) demonstrated a ~1.5-fold increase in the levels of the Munc18c protein level in C2C12-LPL (Fig. 4C).

Both C2C12-control and C2C12-LPL cells demonstrated significant rises in media FFA over the experimental period when exposed to triglyceride-rich media (Fig. 4D). A consistent linear rise in media FFA was observed with C2C12-control cells, whereas, with the C2C12-LPL cells, the rise was initially rapid before beginning to plateau after 4 h. The change in media FFA was significantly greater in C2C12-LPL cultures than in C2C12-control cultures.
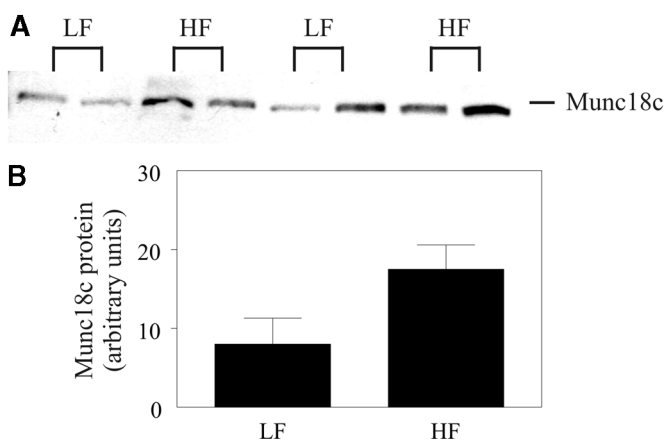

Fig. 3. Munc18c is increased in insulin-resistant mice. Male mice (FVB) were fed either a high-fat (HF) or a low-fat (LF) diet for at least 4 weeks. Results are shown as mean ± SE. A: Munc18c protein levels are increased in the gastrocnemius muscle of mice fed HF compared with LF. B: HF feeding (n = 4) resulted in an ~200% increase in Munc18c protein compared with LF feeding (n = 4). $P = 0.079$, Student's *t*-test.
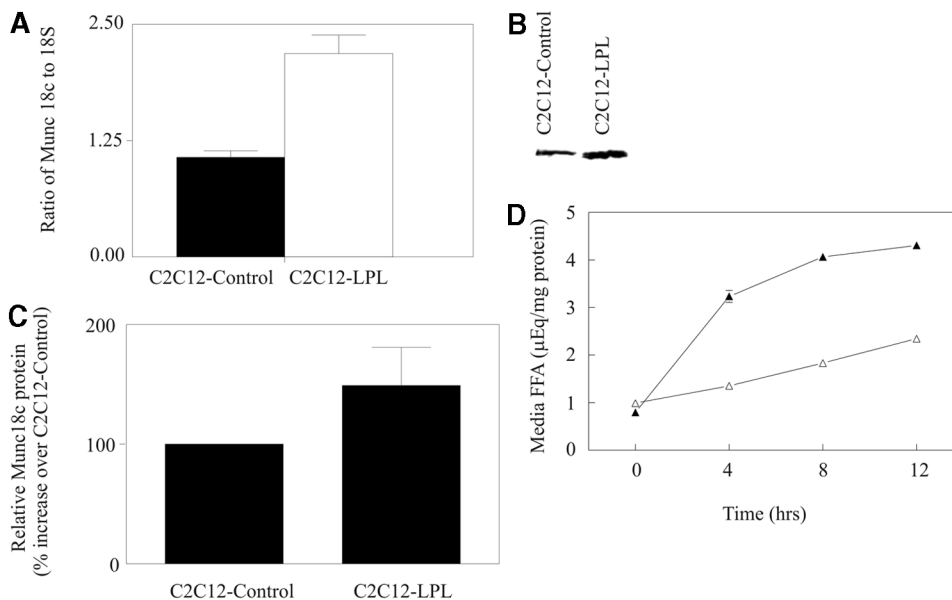

Fig. 2. Munc18c protein in adipose tissue and liver. Tissue extracts from three MCKhLPL and three control mice were prepared from adipose tissue (A) and liver (B) and subjected to SDS-polyacrylamide gel electrophoresis. The samples were then immunoblotted with the Munc18c rabbit polyclonal Ab.

Fig. 4. Munc18c overexpression in C2C12 myocytes transfected with human lipoprotein lipase (C2C12-LPL). Results are shown as mean ± SE. A: RT-PCR analysis for Munc18c transcript in total RNA samples extracted from C2C12-LPL (n = 2) and C2C12-control (n = 2) cells at Day 14 of culture. The standard for RNA input in the RT-PCR reactions was 18S rRNA. B: Representative Western blot analysis of protein extracts from C2C12-control and C2C12-LPL cells at Day 14 of culture. C: Percent increase in Munc18c protein in C2C12-LPL cells. D: Media FFA concentrations, corrected for spontaneous FFA release from substrate at 37°C and standardized by protein concentration, for C2C12-LPL (closed triangles, n = 4) and C2C12-control (open triangles, n = 4) cells when exposed to triglyceride substrate. $P < 0.001$, repeated measures ANOVA.

Run-on assay results

To measure the effects of LPL overexpression on the relative rates of transcription of the Munc18c gene, we performed run-on assays in C2C12-LPL cells. Cells expressing LPL at high and low levels were grown to confluency and their nuclei isolated and incubated with DIG-11-UTP. The labeled transcripts were then hybridized to complete cDNA probes for DGAT, which is unaffected by LPL overexpression in C2C12 cells, a negative control vector, and Munc18c. There was a 1.8-fold increase in the rate of Munc18c transcription in the high LPL-expressing cells when compared with the low-expressing cells (**Fig. 5**). These results parallel our MCKhLPL microarray results, which showed a ~2-fold increase for an EST highly similar to the Munc18 gene.

DISCUSSION

The insulin-sensitive glucose transporter, GLUT4, continuously recycles between the plasma membrane and various intracellular compartments in the basal state. Upon stimulation with insulin, a significant and rapid increase of GLUT4 at the cell surface occurs (7, 22–24). GLUT4 vesicles contain the v-SNARE protein, VAMP2, which interacts with t-SNARE partners at the plasma membrane (syntaxin 4 and SNAP23) during the translocation process. A number of proteins that appear to regulate the assembly of this SNARE complex, such as Munc18c (23),

Synip (23), and Tomosyn (D. E. James, unpublished observations), have been implicated to play an intimate role in regulating GLUT4 trafficking. Although the trafficking of GLUT4 appears to be impaired during insulin resistance, the expression of GLUT4 protein in muscle is not disrupted, suggesting that the defect may occur in one of these regulatory molecules (24).

Sec1p-Munc18 proteins play an important role in regulating the assembly of SNARE complexes. It has been shown in vitro that Munc18 proteins bind to syntaxin and inhibit the ability of syntaxin to interact with its cognate SNARE partners (25, 26). Thus, in this respect, Munc18 proteins are thought to act as negative regulators of SNARE complex assembly. Consistent with this negative role, overexpression of Munc18c in skeletal muscle through adenoviral delivery results in an inhibition of insulin-stimulated glucose transport (9). In contrast, in *Saccharomyces cerevisiae*, deletion of any one of the known four SEC1 alleles results in inhibition of vesicle transport (27). Similarly, Munc18a (28) or Munc18c deletions (29) in mice are lethal. These data would suggest that Munc18 proteins play both a negative and a positive role in vesicle transport. These contradictory findings have partly been resolved by recent studies showing that deletion of the Sec1p-like protein Vps45p in *S. cerevisiae* results in proteasomal degradation of its cognate syntaxin, Tlg2p (30). Munc18c may have a stabilizing influence on syntaxin 4, suggesting that it may be critical for the cell to maintain stoichiometric levels of these proteins (31). Hence, either overexpression or underexpression of Munc18c may have
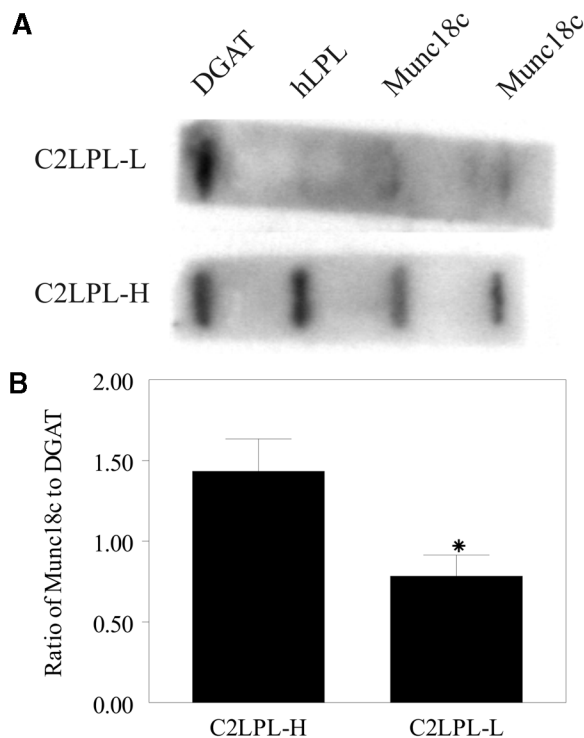
A

C2LPL-L

C2LPL-H

B

Fig. 5. Effect of LPL overexpression on Munc18c gene transcription. Results are shown as mean ± SE. Nuclei from C2C12 cell clones with high (H, n = 3) and low (L, n = 3) LPL activity were used in transcription reactions with DIG-11-UTP label and hybridized to denatured cDNA blots. A: Representative run-on blots for diacylglycerol acyltransferase (DGAT), hLPL, and Munc18c probes. Chemiluminescent detection was carried out with CSPD for 1 h at room temperature. B: Cumulative results of Munc18c run-on assays using the DGAT probe as a standard. The combined data indicate a 1.82-fold increase in Munc18c transcription from C2LPL-H cells. *P = 0.03, Student's t-test.

a deleterious effect on vesicle transport. Although these data suggest that Munc18c might be a key player in the development of insulin resistance, all of the work done on Munc18c has either been done in vitro or through direct manipulation of Munc18c levels.

To date, Munc18c has not been linked with a model of insulin resistance. Our laboratory generated a transgenic mouse model that overexpresses human LPL specifically in skeletal muscle (MCKhLPL) (4). The rationale for producing this model comes from the theory that increases in muscle LPL alter lipoprotein-derived FFA partitioning away from adipose storage to oxidation in muscle. Recent work conducted in our laboratory showed that overexpression of LPL in skeletal muscle leads to an insulin-resistant state (5). The specificity of the up-regulation of the Munc18c gene in MCKhLPL skeletal muscle is reflected in the lack of Munc18c increase in other insulin-responsive tissues, such as adipose tissue and liver. Furthermore, muscle-specific LPL overexpressors generated by Zechner and coworkers (32) have insulin resistance only in the muscle, with normal insulin action in the liver and adipose tissue (33). To further study the connection of Munc18c expression with the lipid-mediated insulin resistance, we carried out Western analysis in muscle biopsies

from high-fat-fed FVB insulin-resistant mice. As anticipated, the Munc18c levels in the high-fat-fed muscle samples were ~2-fold higher than those in the low-fat-fed controls. Altogether, these results underscore the relationship between lipid delivery to the muscle, Munc18c levels, and insulin resistance. When considered in the context of the present study, our work demonstrates for the first time in vivo that insulin resistance is associated with an increase in muscle Munc18c, suggesting that this molecule may play a crucial role as a determinant of whole-body insulin action.

Although the increases observed in mRNA (~1.6-fold) and protein levels (~1.7-fold) of Munc18c in MCKhLPL mice are modest, some investigators propose that Munc18c may function at substoichiometric levels with respect to syntaxin 4 (9, 34, 35). Therefore, small changes in Munc18c content could offset the modulation of GLUT4 vesicle trafficking provided by the Munc18c-syntaxin 4 interaction, especially insofar as no changes in syntaxin 4 and SNAP23 were observed in the MCKhLPL mice. In support of this possibility, a recent report by Fujita et al. (36) showed that despite the increased levels of syntaxins present in rat spleen and kidney compared with liver, they all share a similar molar ratio of syntaxin-Munc18 species. This suggests that this ratio is important in the regulation of vesicle translocation to the plasma membrane. Furthermore, in 3T3-L1 adipocytes, overexpression of syntaxin 4 does not inhibit the insulin-stimulated translocation of the GLUT4 vesicles; in fact, it rescues the inhibition produced by the increase of Munc18c protein (35). SNAP23 is also a possible target for modulation of the vesicle-membrane interaction due to the fact that its overexpression increases insulin action on GLUT4 and glucose uptake in microinjected and chemically permeabilized fat cells (37), although no differences in protein levels were detected between MCKhLPL and control mice. The fact that the mRNA levels of Munc18c are increased but syntaxin 4 mRNA expression is not altered indicates that the Munc18c gene may be a point of regulation in muscle insulin resistance.

We have developed an in vitro model of muscle LPL overexpression using the mouse C2C12 muscle cell line (13) that may enable us to investigate the regulation of Munc18c in more depth. C2C12-LPL cells generate more LPL-derived FFA from triglyceride and accumulate more intracellular lipid over time than do C2C12-control cells and have increased levels of Munc18c mRNA and protein. The increase in Munc18c expression was seen in these cells despite a reportedly low GLUT4 content (38), suggesting that lipid uptake and/or accumulation might be a factor in the regulation of Munc18c expression. In this model, however, it is difficult to link Munc18c protein content with insulin sensitivity, as nearly all cultured muscle cells express little or no GLUT4 (39). In fact, there is no change in glucose uptake in C2C12 myotubes even after inducing an ~10-fold overexpression of GLUT4, indicating that insulin-sensitive vesicle formation and/or transport does not take place in these cells (40).

To gain a better understanding of the nature of the Munc18c-increased expression in C2C12-LPL cells, we turned to nuclear run-on experiments that were designed

to measure the effects of the LPL overexpression on the relative rates of transcription of Munc18c and other genes. The run-on results indicated that LPL overexpression in C2C12 cells mediated its effects on Munc18c through increased Munc18c gene transcription. Thus, the excess lipid available to the C2C12-LPL cells through the higher LPL enzymatic activity could be responsible for the increase in Munc18c gene transcription.

It is well documented that a link exists between FFA and insulin resistance in skeletal muscle. Specifically, FFA reduces insulin-stimulated glucose transport activity (10, 41). In this respect, recent reviews (2, 23, 42, 43) have proposed an attractive mechanism for FFA-induced insulin resistance in muscle, in which the excess delivery of FFA leads to an increase in intracellular fatty acid metabolites, such as fatty acyl CoA, diacylglycerol (DG), and ceramide. DG activates many isoforms of protein kinase C, leading to phosphorylation of serine-threonine sites on insulin receptor substrates, causing a decrease in phosphatidylinositol 3-kinase (PI 3-kinase) activation, which is necessary for downstream events leading to glucose transport activation. Ceramide does not inhibit insulin-stimulated glucose transport through decreased PI 3-kinase activation, but does so downstream through decreased phosphorylation and activation of Akt-protein kinase B (44, 45). Another mouse model of muscle LPL overexpression with insulin resistance (33) showed significant accumulation of long-chain fatty acyl CoA, ceramide, and DG. Thus, defects in insulin signaling and action are associated with increased lipid-derived metabolites in muscle.

The downstream effects of the defective PI 3-kinase signaling cascade on the glucose transport machinery is obscure. The possibility remains that fatty acid metabolites regulate the SNARE and accessory proteins (e.g., Munc18c) independently but in parallel to the PI 3-kinase pathway. For either mechanism, alone or in tandem, the ultimate result of increasing the delivery of fatty acids to muscle would be defects in insulin-mediated glucose transport.

In conclusion, our data demonstrate for the first time in vivo that an insulin-resistant state brought about by lipid delivery to skeletal muscle is associated with an increased transcription rate and protein content of the Munc18c gene. This up-regulation of Munc18c may in part be responsible for the muscle insulin resistance observed in MCKhLPL mice.

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