Fatty acids increase glucose uptake and metabolism in C2C12 myoblasts stably transfected with human lipoprotein lipase

Warren H. Capell,1 Isabel R. Schlaepfer,1 Pamela Wolfe,3 Peter A. Watson,1 Daniel H. Bessesen,1 Michael J. Pagliassotti,2 and Robert H. Eckel1

1Division of Endocrinology, Metabolism, and Diabetes, University of Colorado at Denver and Health Sciences Center, Denver; 2Department of Food Science and Human Nutrition, Colorado State University, Fort Collins; and 3Department of Preventive Medicine and Biometrics, University of Colorado at Denver and Health Sciences Center, Denver, Colorado

Submitted 8 October 2009; accepted in final form 5 July 2010.

Fatty acids can compete as oxidative fuels within muscle for review, see Randle (37). Additionally, increased triglyceride (TG) content has been associated with insulin resistance in various insulin-sensitive tissues (21, 24, 29, 31, 41, 43), and accumulation of lipid species within cells might directly affect cell insulin signaling (42). Therefore, examining cellular interactions between lipid and glucose may be valuable in understanding cell metabolism and the pathogenesis of insulin resistance. Studies of the cellular effects of lipids typically focus on free fatty acids (FFA) and have only rarely examined lipoprotein TG-derived fatty acids (TGFA). Circulating within lipoproteins, TGFA have the potential for unique cellular interactions that are distinct from FFA. Specifically, TGFA enter skeletal muscle cells and adipocytes following hydrolytic release, requiring the hydrolytic enzyme lipoprotein lipase (LPL) (14). Additionally, the binding of lipoproteins to LPL at the endothelial surface allows lipoproteins to interact with cell receptors and promotes whole particle uptake (6, 13, 28, 38). Most studies to date utilizing TG emulsions have coinfused heparin, which releases LPL from its endothelial-bound position and disrupts these potential bridging functions. Because of the inclusion of heparin in previous studies, cellular effects of TGFA via metabolism by intact LPL have not been adequately examined.

We have previously described C2C12 myocytes stably transfected with human LPL to examine metabolic consequences of cellular TGFA overexpression (33). These cells accumulate TG during proliferation and have the potential for acutely increased delivery of TGFA when exposed to TG-rich particles. Because GLUT4 expression in the C2C12 cell line is low (22, 45), these cells are best suited to assess metabolic effects independent of insulin stimulation. The aim of the present study was to examine the relationship between LPL expression, TG availability, and glucose metabolism in C2C12 myoblasts. We hypothesized that, in the absence of insulin, glucose utilization would be impaired when TG fuel was provided to myoblasts, particularly in cells overexpressing LPL.

MATERIALS AND METHODS

Cell culture. Details concerning the transfection and selection of C2C12 myoblast lines have been published previously (33). The following studies utilized these C2C12 myoblasts stably transfected with a retroviral vector containing human LPL cDNA (C2/LPL) and control myoblasts transfected with a fusion gene of β-galactosidase and neomycin phosphotransferase. The cell lines have been characterized previously, demonstrating >15-fold greater cell surface and heparin-releasable LPL activity in C2/LPL cells compared with controls (33). All cells used in the present study were of passage 5 to 7.

Control and C2/LPL myoblasts were plated separately at initial densities of ~3.0 × 103 cells/well on 35-mm plates and grown at 37°C in an atmosphere of 95% air-5% CO2 at 100% humidity. Cultures were grown to confluence in high-glucose DMEM (Invitrogen, Grand Island, NY) supplemented with 20% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 200 mM L-glutamine solution (Invitrogen), penicillin-streptomycin solution (Invitrogen), and 320 μg/ml G418 (Invitrogen). The high-medium serum content kept cells in a proliferative, nondifferentiated state. Culture medium was refreshed within 24 h prior to any experimental procedure.

LPL activity and assessment of LPL inhibition by tetrahydro- lipstatin. Culture medium was aspirated from duplicate wells of confluent myoblasts, and cells were washed three times with ice-cold Krebs-Ringer phosphate buffer (KRP). Cell-surface LPL was released by incubating parallel control and C2/LPL cultures with ice-cold KRP containing 15 μg/ml sodium heparin and increasing concentrations (vehicle only, 5 μM, and 50 μM) of the LPL catalytic site inhibitor tetrahydro-lipstatin (THL) (25) for 5 min with gentle rocking on ice.
THL was kindly provided by Drs. M. K. Meier and P. Weber (F. Hoffman-La Roche, Basel, Switzerland). LPL activity was assayed in duplicate samples of supernatant by previously described methods (15).

**Media preparation and myoblast incubation.** Experimental media were prepared from a base medium consisting of FBS-free, low-glucose DMEM supplemented with d-glucose (Sigma, St. Louis, MO) to produce a final glucose concentration of 12 mM. In the subset of experiments examining intracellular glucose retention, [U-14C]glucose (American Radiolabeled Chemicals, St. Louis, MO) was added to this base medium (final specific activity = 42 μCi/ml).

From this base medium, four separate isovolemic media were created, each containing fatty acid-free albumin solution and a small aliquot (2.3% vol/vol of fasting human serum to provide apoC-II activator for LPL, and 1) TG− medium contained additional KRP only, 2) TG+ medium contained Intralipid-20 (20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin; Baxter, Deerfield, IL) with a final TG concentration of 55 mg/dl, and 3) FFA medium contained albumin-bound oleic acid (Sigma) with a final concentration of 1,200 μM and a 3:1 molar ratio of oleate to albumin. FFA medium was designed to approximate peak FFA concentrations observed during TG+ treatment to evaluate the effects of FFA independent of TG particles, and 4) THL medium was prepared identically to TG+ medium but also contained THL in a final concentration of 50 μM to evaluate the role of LPL-mediated hydrolysis. No insulin or FBS was added to these media.

Confluent control and C2/LPL cultures were incubated for 12 h with each of the four different experimental media. Media were sampled at baseline and at 4-h intervals (total of 5% medium volume removed for sampling), and media from the duplicate wells were pooled for analyses in each independent experiment. In the subset of experiments that included labeled glucose tracer, cells were harvested for analyses at the end of incubation. Media were aspirated, cells were washed three times with ice-cold KRP, and cells from each of the duplicate wells were harvested separately in KRP and 0.03 N HCl for extraction of lipid and glycogen, respectively.

For experiments using 14C-labeled glucose, total cell lipids were extracted from cells using the Bligh and Dyer (7) method. Lipid extracts were then separated into subfractions using the solid-phase extraction method of Kaluzny et al. (20), modified with increased volumes of solvents to optimize yield. Each subfraction was counted to determine 14C label retention. Cell glycogen radiolabel retention was determined by the methods of Chan and Exton (12).

**Oil red O staining.** Parallel control and C2/LPL myoblast cultures were incubated with media as described above. At the end of incubation, cells were washed three times with ice-cold KRP and fixed in 4% paraformaldehyde. Cells were then washed three times with KRP and stained with Oil red O solution (60% isopropanol) for 10 min. The stain was removed, and the cells were washed briefly with 60% isopropanol and subsequently with KRP prior to microscopy. Densitometry of micrographs was assessed using AlphaEaseFC software (version 4.0).

2-Deoxyglucose uptake. Parallel control and C2/LPL myoblast cultures were incubated with cell growth medium containing 1,000 μM olate-albumin or vehicle (BSA). After 4 h, identical media containing 0.1 mM 2-deoxyglucose and 1 μCi/ml 2-[3H]deoxyglucose were introduced, and cells were incubated for 20 min. Media were removed, cells were washed three times with KRP, and cells were harvested to determine 3H retention.

**Western blotting.** Parallel control and C2/LPL myoblast cultures were incubated with media described above and harvested in cell lysis buffer containing 20 mM Tris, 150 mM NaCl, 1% NP-40, 20 mM NaF, 2 mM EDTA, 2.5 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10% glycerol, and protease/phosphatase inhibitors [Pefabloc SC (Roche); Complete, Mini, EDTA-free (Roche); and phosphatase inhibitor cocktail 2 (Sigma)] cells were lysed for 45 min at 4°C with gentle rocking and centrifuged for 12 min at 10,000 g at 4°C to remove cell debris.

Cell proteins were separated on 10% polyacrylamide gels in sodium dodecyl sulfate-Tris-glycine buffer and transferred to polyvinylidene difluoride membranes for immunodetection. Membranes were blocked in 5% nonfat milk (5% BSA for hexokinase II) in TBST (Tris-buffered Saline with 0.1% Tween 20) before incubation with primary antibodies. Primary antibodies against phosphorylated (no. 4058; Ser473) and total (no. 9272) Akt, phosphorylated (no. 2531; Thr172) and total (no. 2532) AMP-activated kinase (AMPK), and hexokinase II (no. 2867) were purchased from Cell Signaling Technology (Danvers, MA). Primary antibodies against GLUT1 (no. sc-7903) and GLUT4 (no. 4670–1709) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Biogenesis (Kingston, NH), respectively. Membranes were washed with TBST and incubated with antigen-specific antibodies (anti-rabbit and anti-mouse, Cell Signaling Technology; anti-goat, Santa Cruz Biotechnology), and antibody target protein complexes were detected with enhanced chemiluminescence (CDP-Star; New England Biolabs, Ipswich, MA). For GLUT1 (n = 3; 70 μg of protein), GLUT4 (n = 6; 70 μg), and hexokinase II (HKII; n = 4; 70 μg), membranes were stripped and reprobed with anti-β-actin antibody (Sigma) as a loading control. For Akt (n = 5; 100 μg) and AMPK (n = 3; 70 μg), membranes were reprobed for phosphorylated protein and then stripped and reprobed for total protein. Autoradiographic films were analyzed densitometrically using a Fluor-S Multimager and Quantity One software (Bio-Rad Laboratories, Hercules, CA).

**Statistical analysis.** All results shown are expressed as means ± SE. Analyses were performed using SPSS software (version 14.0; SPSS, Chicago, IL). Results were compared for overall treatment effects between four treatments using either one-way ANOVA or multivariate ANOVA (MANOVA) (17) where appropriate. MANOVA was utilized to test the effects of treatments on glucose retention in individual cell lipid subfractions, between four levels of treatment and seven subfraction measurements for each treatment, and was assessed for significance using Roy’s greatest root. A similar approach was used to test the effects of treatment on potential mediators of glucose uptake between four levels of treatment and two cell types. Within each measurement, the treatment contrasts for TG− vs. TG+ and FFA and TG+ vs. FFA and THL were estimated based on a two-tailed t-test in an ANOVA model. Contrasts are presented only for when the main effect for treatment was significant, thus controlling the type I error rate within measurements. The level of significance was α = 0.05.

**RESULTS**

**Cultured myoblasts and TGFA delivery.** Heparin-releasable LPL activity in C2/LPL cultures was 15-fold higher than in controls and was readily inhibited by THL (Fig. 1A). Because 50 μM was the minimum concentration of THL found to completely inhibit LPL activity in both cell types, this concentration was used for all THL experiments. Figure 1B illustrates the interaction of cell LPL in situ with Intralipid substrate. Medium FFA concentration rose when C2/LPL cells were exposed to TG+ medium, an effect blocked by the presence of THL (P < 0.001). In control cultures, TG+ medium caused a small increase in medium FFA concentration relative to treatment with THL medium (P = 0.05). Both control and C2/LPL myoblasts accumulated cellular lipid when incubated with FFA medium; only C2/LPL myoblasts had significant intracellular neutral lipid accumulation with TG+ treatment (Fig. 2A and Supplemental Fig. S1; Supplemental Material for this article).
can be found online at the AJP-Endocrinology and Metabolism web site). Cell viability assays (Vi-CELL XR2.03; Beckman Coulter, Brea, CA) confirmed myoblast viability in both control (96±H11001 1.1%) and C2/LPL (97±H11006 0.9%) cells following 12-h incubations with FFA medium.

Medium glucose metabolism with TGFA and FFA exposure. Treatments had no effect on medium glucose disappearance in control cells (Fig. 2A). Medium glucose disappearance was significantly increased in C2/LPL cells in the presence of TG (P < 0.001) or FFA (P = 0.001) but did not differ between these treatments.

We hypothesized that the increased glucose disappearance seen in C2/LPL cells under TG+ and FFA conditions occurred due to increased influx of fatty acids for subsequent assembly of TG within these cells. To test this hypothesis, we assessed [U-14C]glucose label retention in cell lipids under the different experimental conditions (Fig. 3). Under TG− conditions, the majority of medium glucose retention in cell lipid was in the
phospholipid pools in both control and C2/LPL cells. Treatment with TG or FFA increased retention of glucose label in cell TG in both cell types. In control cells, glucose retention in cell TG was greater with FFA compared with TG treatment ($P < 0.001$). Retention of glucose label in cell phospholipids showed no significant differences across treatments in either control or C2/LPL cells. No differences were observed in glucose label retention in glycogen between treatments in either cell type (Supplemental Fig. S3), demonstrating specificity of this increased retention for the cell TG pools.

**Role of LPL-mediated hydrolysis.** Based on the above results, we hypothesized that the increased glucose disappearance in C2/LPL cells under TG+ conditions was dependent on TGFA liberation by lipolysis with subsequent cell TG synthesis. To test this hypothesis, cells were exposed to Intralipid in the presence of THL (Fig. 4). In control cells, THL did not have significant effects on glucose disappearance (Fig. 4A) or glucose label accumulation in cell TG ($P = 0.08$ vs. TG+; Fig. 4B). In C2/LPL cells, THL significantly reduced medium glucose retention in cell TG ($P = 0.004$; Fig. 4B) but did not significantly alter glucose disappearance ($P = 0.08$ vs. TG+; Fig. 4A). Glucose disappearance remained elevated in C2/LPL cells in the presence of THL relative to TG− conditions ($33.9 \pm 1.7$ vs. $28.3 \pm 1.3 \, \text{pmol/well}$, $P = 0.03$), confirming that inhibition of LPL hydrolysis did not inhibit the increased glucose uptake in the presence of Intralipid. The increased glucose disappearance with THL treatment was not accounted for by altered retention in cell glycogen (Supplemental Fig. S3).

Because glucose disappearance and glucose retention in C2/LPL cell TG were discordant in the presence of THL, Oil red O staining was performed (Fig. 4C and Supplemental Fig. S3). In C2/LPL cells, THL did not significantly inhibit cell neutral lipid accumulation ($P = 0.09$), which remained increased compared with TG− conditions ($P = 0.02$).

**Potential mediators of glucose uptake.** To examine whether the observed changes in glucose disappearance could be due to changes in glucose transport or phosphorylation, we assessed changes in 2-deoxyglucose uptake, potential mediators of glucose transport, and HKII.

FFA treatment did not significantly alter 2-deoxyglucose uptake in either C2/LPL ($41.4 \pm 4.2$ vs. $29 \pm 45.6 \, \text{pmol/ml}^{-1} \cdot \text{mg}^{-1}$, vehicle vs. FFA, $P = 0.1$) or control ($37.0 \pm 6.2$ vs. $38 \pm 8.4 \, \text{pmol/ml}^{-1} \cdot \text{mg}^{-1}$, vehicle vs. FFA, $P = 0.9$) cells. Although C2/LPL cells had significantly lower Akt phosphorylation ($P = 0.01$) and HKII expression ($P = 0.03$), no acute changes in these proteins, AMP-activated protein kinase, or cell GLUT1 or GLUT4 content were observed to explain the increased glucose disappearance in the LPL-overexpressing cells when lipid was present in the medium (Fig. 5).

**DISCUSSION**

In the present study, we found that medium lipid availability results in increased medium glucose label retention in cell TG of cultured myoblasts. This increase occurred in the presence of both FFA and TGFA in both cell types, although the magnitude of TGFA effect was dependent on activity of cell LPL. These results are not surprising since the glycerol backbone for acyl-glyceride synthesis in muscle and adipose tissue is thought to derive largely from extracellular glucose (19, 36). The lack of increased label incorporation into glycogen storage pools argues the specificity of this relationship for TG synthesis. Therefore, the results support this relationship between extracellular glucose utilization and cell TG synthesis in our cells.

In the present study, we also found that medium lipid availability increases medium glucose disappearance, independent of insulin, in cells that overexpress LPL. One might expect that glucose uptake was increased to provide glycerol for accelerated cell TG synthesis in C2/LPL cells. However, increased glucose disappearance persisted in the presence of THL, whereas glucose label retention in cell TG was inhibited. These findings suggest that lipid availability increases glucose uptake independently of TG synthesis in these cells.

It is noteworthy that only C2/LPL cells demonstrated increased glucose uptake in the presence of medium lipids. In vitro studies have previously examined basal glucose uptake in muscle cells following preincubation with TGFA (30) or FFA (1, 40, 44). Similar to our results in control cells, these studies found little effect on basal glucose transport following such pretreatment, with the exception of eicosapentanoic acid (1). A major difference in our experiments is that glucose uptake was increased to provide glycerol for accelerated cell TG synthesis in C2/LPL cells. However, increased glucose disappearance persisted in the presence of THL, whereas glucose label retention in cell TG was inhibited. These findings suggest that lipid availability increases glucose uptake independently of TG synthesis in these cells.
disappearance was assessed during lipid exposure rather than following lipid pretreatment, and its disappearance was integrated over relatively long periods of time. Short-term measurements of glucose uptake may not be sensitive enough to detect these subtle differences. Additionally, since our control cells did not demonstrate increased glucose uptake during lipid treatments, our results indicate that overexpression of LPL in this myoblast system leads to unique metabolic changes in these cells.

We have previously demonstrated some metabolic differences between C2/LPL and control cells. C2/LPL cells have increased intracellular TG accumulation (33) and expression of the insulin resistance-associated protein Munc-18c (39) at the time of confluence compared with controls. In the present study, we have also found decreased Akt phosphorylation and HKII expression in C2/LPL cells. These observations provide further evidence that C2/LPL and control myoblasts are metabolically distinct in the basal, untreated state. Therefore, the observed increased glucose uptake in C2/LPL myoblasts is not likely the result of LPL overexpression per se; rather, it results from chronic metabolic changes.

The metabolic changes responsible for our findings remain unclear. Our results do not support changes in either glucose transport or phosphorylation to explain the increased glucose uptake in C2/LPL cells. We postulate that the observed increase in glucose uptake results from metabolic changes, downstream of transport and phosphorylation, that pull glucose into cells through existing membrane transporters.

An important limitation of this study is that we focused our examinations on myoblasts as opposed to myotubes, which might be more physiologically similar to skeletal muscle. Because these stably transfected cells overexpress LPL throughout their growth and differentiation, longer growth periods should lead to chronic metabolic effects of the LPL overexpression, such as those associated with chronic lipid loading. We chose to study less differentiated myoblasts to limit the impact of these chronic metabolic changes and to isolate the effects of overexpressing LPL. However, as discussed above, our data demonstrate that C2/LPL and control cells are already metabolically distinct at the myoblast stage, and our results must be attributed to chronic cellular alterations.

Another important limitation in the present study is that LPL in these cells is expressed at the myocellular surface instead of at the surface of an endothelial layer, as is typical in vivo. The lack of an endothelial layer in our system could have contributed to the significant cell lipid accumulation by Oil red O with THL treatment, which appears to have occurred independently of increased TG synthesis. Such accumulation could result from inhibition of intracellular lipolysis and/or an increase in whole particle uptake. It is possible that the lack of an endothelial layer increased the availability of Intralipid particles for uptake by cells. LPL promotes uptake of lipoprotein core lipids (27) but can do so even when anchored to the subendothelial myocellular surface (47). Furthermore, TG-rich lipoproteins can enter the subendothelial space in vivo (35). Therefore, it is not clear whether this physiological difference limits the ability to generalize our results; regardless, it should not account for the differences observed between cell types.

Whereas in vivo human studies have consistently shown that elevated FFA inhibit glucose uptake in the presence of insulin stimulation (3, 8, 9, 11, 16, 23, 32, 48), increased glucose uptake (48), decreased uptake (9, 32), and no effect (3, 8, 16) of FFA have all been reported in the absence of increased insulin stimulation. Our results illustrate a model in which
basal glucose uptake can be modified favorably in the presence of lipids. Exploitation of alternate pathways of glucose uptake could be valuable in treating disorders with impaired insulin-mediated uptake. Our C2/LPL myoblasts have some characteristics reported previously in insulin-resistant states such as increased myocellular lipid accumulation, increased non-insulin-mediated glucose uptake, decreased Akt phosphorylation, and decreased hexokinase II expression. Intracellular lipid accumulation with concurrent increased glucose uptake is also a characteristic reported in cancer cell models. Given these metabolic similarities, our cells could provide an additional model to examine metabolic alterations common to cancer and insulin resistance disorders.

In summary, we have demonstrated exposure to TG-rich particles and FFA result in increased glucose disappearance and metabolism, independent of insulin, in stably transfected myoblasts overexpressing lipoprotein lipase. This effect does not depend on the hydrolysis of TG with subsequent generation...
of FFA and might relate to chronic metabolic adaptations in cells with lipid accumulation. A more thorough understanding of the cellular cross-talk between lipids and glucose is needed.

ACKNOWLEDGMENTS

We thank Carrie McCurdy, Dwight Klemm, Leslie Knaub, Jennifer Yoon, and David Gordon for assistance and meaningful discussions.

GRANTS

This work was supported by National Institutes of Diabetes and Digestive and Kidney Diseases Grants DK-69291 (W. H. Capell), DK-26356 (R. H. Eckel), DK-47416 (M. J. Pagliassotti), and DK-02935 (D. H. Bessesen).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

37. Salinelli S, Lo JV, Mims MP, Zsigmond E, Smith LC, Chan L. Structure-function relationship of lipoprotein lipase-mediated enhancement of very low density lipoprotein binding and catabolism by the low density lipoprotein receptor. Functional importance of a properly folded


