Glycerol is taken up by human muscle in vivo and incorporated into lipids, but little is known about regulation of glycerol metabolism in this tissue. In this study, we have analyzed the role of glycerol kinase (GIK) in the regulation of glycerol metabolism in primary cultured human muscle cells. Isolated human muscle cells exhibited lower GIK activity than fresh muscle explants, but the activity in cultured cells was increased by exposure to insulin. [U-14C]Glycerol was incorporated into cellular phospholipids and triacylglycerides (TAGs), but little or no increase in TAG content or lactate release was observed in response to changes in the medium glycerol concentration. Adenovirus-mediated delivery of the Escherichia coli GIK gene (AdCMV-GIK) into muscle cells caused a 30-fold increase in GIK activity, which was associated with a marked rise in the labeling of phospholipid or TAG from [U-14C]glycerol compared with controls. Moreover, GIK overexpression caused [U-14C]glycerol to be incorporated into glycogen, which was dependent on the activation of glycogen synthase. Coincubation of AdCMV-GIK-treated muscle cells with glycerol and oleate resulted in a large accumulation of TAG and an increase in lactate production. We conclude that GIK is the limiting step in muscle cell glycerol metabolism. Glycerol 3-phosphate is readily used for TAG synthesis but can also be diverted to form glycolytic intermediates that are in turn converted to glycogen or lactate. Given the high levels of glycerol in muscle interstitial fluid, these findings suggest that changes in GIK activity in muscle can exert important influences on fuel deposition in this tissue.

Glycerol levels in human muscle interstitial fluid are much higher than plasma levels and approach the concentrations found in adipose tissue (1, 2). This pool of glycerol may be important for synthesis of TAG1 in muscle. Importantly, recent studies have shown that intramuscular overstorage of TAG is closely correlated with muscle insulin resistance (3, 4). Nonetheless, there is little information about the regulation of muscle TAG synthesis, which depends on the provision of exogenous fatty acids (5) and the availability of glycerol 3-P for esterification.

Glycerol 3-P can be derived from direct uptake of glycerol and its phosphorylation by GIK or, alternatively, as a byproduct of the glycolytic pathway. Demonstration of significant extraction of glycerol by forearm muscle has been reported, whereas no uptake was detected in adipose tissue (6). Moreover, studies of incorporation of glycerol into muscle TAG showed that it was comparable to that of glucose on a carbon-equivalent basis in fed rats and much greater than that of glucose in fasted animals (7). GIK activity has been detected in rat (8) and human (9) skeletal muscle. The activity is higher in rat red than in white muscle (8), although activity levels in either muscle fiber type are much lower than in liver. This is consistent with the finding of abundant GIK mRNA in liver, kidney, and testis in human and mouse and much lower transcript levels in muscle (10). It has been suggested that liver and muscle express different GIK genes based on the dissociation between deficiencies in liver and muscle GIK activity in human patients (9).

The current study was undertaken to evaluate the role of GIK in control of glycerol metabolism in muscle cells. While low rates of glycerol conversion to cellular products can be demonstrated in cultured human myocytes, these variables are only modestly affected by altering the medium glycerol concentration. However, overexpression of GIK in these cells caused large, glycerol concentration-dependent increases in accumulation of cellular lipids, glycogen, and lactate. Thus, these studies demonstrate that all of the enzymes required for glycerol utilization in several different pathways are abundantly expressed in muscle cells and that glycerol metabolism is controlled by the level of GIK. These findings provide a new perspective on the potential relevance of glycerol to muscle metabolism, particularly under catabolic circumstances where glycerol levels are elevated.

EXPERIMENTAL PROCEDURES

Human Muscle Primary Cultures and Transduction with Adenovirus—Human muscle primary cultures were initiated from a bank of satellite cells of muscle biopsies obtained from patients considered free of muscle disease (biopsies were obtained with informed consent and approval of the Human Use Committee of Hospital Vall d’Hebron, Barcelona, Spain). Aneural muscle cultures were established in a monolayer through an explant-reexplantation technique as described by Askanas et al. (11). Cultures were grown in a Dulbecco’s modified Eagle’s medium-M199 medium (3:1) supplemented with 10% fetal bovine serum, 10 μg/ml insulin (Sigma), 2 mM glutamine (Sigma), 25 ng/ml fibroblast growth factor, 10 ng/ml epidermal growth factor (Becton Dickinson, Franklin Lakes, NJ). Immediately after myoblast fusion,

2682 This paper is available on line at http://www.jbc.org
cells were rinsed in Hank's balanced salt solution, and a medium devoid of fibroblast growth factor, epidermal growth factor, and glutamine was added. Muscle cultures were maintained in this medium for up to 2 weeks.

Construction and use of recombinant adenoviruses containing the bacterial GIK gene (AdCMV-GIK) or the cDNA encoding a glycogen-ta

ging subunit of protein phosphatase-1 known as protein targeting subunit of protein phosphatase-1 (AdCMV-PTG) have been described elsewhere (12, 13). Control virus (AdCtrl) contained the expression cassette backbone with no insert. The adenoviruses were amplified in 293 cells, and viral stocks of 7 × 10^9 plaque-forming units/ml were prepared in 10% fetal bovine serum, Dulbecco's modified Eagle's medium as described previously (14). Gene delivery to muscle cultures was achieved by exposing myotubes, induced to fuse by removal of growth factors, to the virus for 2 h at a multiplicity of infection of 10.

Preparation of Solutions Containing Fatty Acids—Sodium salt of oleic acid (Sigma) was prepared immediately prior to utilization by dissolving the fatty acid in deionized water containing 1.2 eq of NaOH at 70 °C with stirring until an optically clear dispersion was obtained. The fatty acid-salt solution was immediately added to Dulbecco's modified Eagle's medium containing fatty acid-free bovine serum albumin (BSA) (Sigma) with continuous agitation to avoid precipitation. The fatty acid/BSA molar ratio was 5:1.

Glycerol Kinase Activity Assay—To measure enzyme activities, extracts were prepared by scraping cell monolayers into a buffer consisting of 50 mM HEPES (pH 7.8), 40 mM KCl, 11 mM MgCl₂, 1 mM EDTA, and 1 mM diethylene triamine pentaacetic acid (DTPA). The homogenates were centrifuged at 10,500 × g for 15 min, and the resulting supernatants were used for the determination of GIK activity. GIK activity was measured using a spectrophotometric assay (Bio-Rad Protein Assay Reagent) in a Cobas Fara II autoanalyzer. Protein concentration was measured using the Bio-Rad protein assay reagent.

Metabolite Determinations—Glycerol-3-P was measured enzymatically in neutralized HClO₄ extracts. Lactate concentration in the incubation medium was determined enzymatically.

Acylglyceride Determination—To determine the TAG content, extracts were prepared by scraping cell monolayers into a buffer consisting of 50 mM Tris, 100 mM KCl, 20 mM KF, 0.5 mM EDTA, 0.05% Lubrol PX, pH 7.9 and three rounds of sonication for 5 s each (16). Homogenates were centrifuged at 11,000 × g for 15 min, and the resulting supernatants were collected. Protein concentration was measured with the aid of Bio-Rad protein assay reagent. Total TAG were measured enzymatically with a Cobas Fara II autoanalyzer with a GPO-Trinder (Sigma) kit using triolein resuspended in the extraction buffer as a standard.

Incorporation of [U-¹⁴C]Glycerol into Cellular Lipids: Thin-layer Chromatography Analysis—Cells were incubated with [U-¹⁴C]glycerol (100 μCi/mmole) (Amersham Biosciences, Inc.) and 5 mM glucose for 15 h. The cell monolayers were then washed in phosphate-buffered saline, scraped into 100 μl of 30% KOH, and boiled for 15 min. An aliquot of the homogenates was used for measurement of protein concentration. Homogenates were spotted onto Whatman 31ET paper, and glycerol was precipitated by immersing the papers in ice-cold 66% ethanol. Dried papers containing precipitated glycerol were suspended in scintillation fluid for measurement of incorporated radioactivity.

Statistics—Differences between groups were assessed by Student’s t test.

RESULTS

Glycerol Kinase Activity—GIK activity was measured in cultured human muscle cells exposed to a control virus (AdCtrl). Enzyme activity measured in 15-day cultures of human myotubes was only 25% of that found in freshly isolated gastrocnemius muscle from fed rats (5.1 ± 0.3 versus 21 ± 1.6 milliunits/mg of protein, respectively). Since insulin is known to increase GIK activity in hepatocytes (17), we examined its potential effect in cultured human muscle cells. Incubation with 100 nM insulin for 15 h caused an 80% increase in GIK activity (to 9.2 ± 1 milliunits/mg of protein). To further increase GIK activity, human muscle cells were treated with a recombinant adenovirus containing the GIK gene (AdCMV-GIK) causing a 30-fold increase in enzyme activity 1 week after viral treatment (to 142 ± 11 milliunits/mg of protein), overexpression that is inherent in the adenovirus-mediated system.

Glycerol 3-P Concentration—Glycerol 3-P levels were determined in control cells incubated with varying glycerol concentrations or other glycogenic precursors such as glucose or lactate. In glucose-deprived cells, no significant changes in glycerol 3-P were detected as a function of varying the glycerol concentration over the range of 0–5 mM (Fig. 1A). Likewise, incubation with 15 mM lactate, a putative glycogenic substrate, did not raise glycerol 3-P levels (0.13 ± 0.02 μg/mg of protein). In contrast, addition of 25 mM glucose doubled glycerol 3-P concentrations relative to glucose-deprived cells (Fig. 1A).
The further addition of glycerol caused a similar small concentration-dependent rise in glycerol 3-P concentrations as in glucose-deprived cells. In sharp contrast to these findings, overexpression of GlK by AdCMV-GlK treatment (Fig. 1B) caused glycerol 3-P levels to rise up to 300-fold in response to extracellular glycerol. In these cells, concomitant addition of glycerol and glucose further increased glycerol 3-P at all doses of glycerol tested. Finally, in control cells treated with insulin, which display higher GlK activity, addition of 5 mM glycerol in the absence of glucose caused a 25% increase (p < 0.05) in the levels of the phosphorylated metabolite (to 0.19 ± 0.02 μg of glycerol 3-P/mg of protein) relative to cells incubated at 0 mM glycerol.

**Triacylglyceride Accumulation** — TAG content was analyzed in cells incubated with varying glycerol concentrations in the presence or absence of 1 mM sodium oleate or 25 mM glucose (Fig. 2). As expected, minimal TAG content was found in control or AdCMV-GlK-treated cells incubated in a medium containing oleate but devoid of glycerol or glucose (Fig. 2, A and C). Control cells incubated without glucose but with glycerol showed a minimal capacity for fatty acid esterification as assessed by lack of increase in their TAG content after addition of oleate (Fig. 2A). Addition of glucose to control cells unveiled a 3-fold increase in TAG levels in response to oleate addition with further increases in TAG of 20% (p < 0.05) upon addition of glycerol (Fig. 1A). GlK-overexpressing cells responded quite differently to this same array of maneuvers (Fig. 2, C and D). Glucose-deprived AdCMV-GlK-treated cells exhibited a large increment in TAG levels in response to oleate, which occurred in a glycerol concentration-dependent fashion, whereas no significant increase was observed in cells incubated with glycerol in the absence of oleate. When GlK-overexpressing cells were incubated with 25 mM glucose and oleate, maximal TAG accumulation was achieved, which was further elevated by provision of glycerol (Fig. 2D).

**Incorporation of [U-14C]Glycerol into Cellular Lipids** — To determine whether glycerol 3-P was being incorporated into cellular lipids, cells were incubated with 5 mM [U-14C]glycerol for 15 h in the presence of 5 mM glucose and 1 mM sodium oleate (Fig. 3). Control cells exhibited detectable incorporation of radioactivity from [U-14C]glycerol into both PL and TAG fractions, indicating that these cells are able to metabolize glycerol and use it for TAG synthesis, albeit at a very low rate. Addition
of insulin caused a 50% increase in incorporation of labeled glycerol into the PL fraction but only a minor increase in labeling of TAG. Again, GIK overexpression had a major impact on glycerol metabolism, resulting in increases of 19- and 21-fold in glycerol incorporation into TAG and PL, respectively, relative to control cells with normal GIK levels.

Incorporation of [U-14C]Glycerol into Glycogen—We next evaluated whether glycerol 3-P could be used to synthesize glucose by measuring the incorporation of [U-14C] glycerol into glycogen (Fig. 4). The radioactivity incorporated into glycogen in control cells incubated with 5 mM glucose and 5 mM [U-14C] glycerol was negligible as it was after incubation with 5 mM glucose and 5 mM [U-14C] lactate (200 μCi/mmol) (data not shown). Moreover, the incorporation of radioactivity was not increased in either glycerol- or lactate-incubated cells by activation of glycogen synthase, the rate-limiting enzyme for glycogen synthesis, through overexpression of PTG (18). In contrast, overexpression of GIK by AdCMV-GIK treatment caused an 8-fold increase in glycerol incorporation into glycogen. Furthermore, when glycogen synthase was activated by concomitant overexpression of PTG, glycerol incorporation into glycogen was doubled.

Lactate Production—In glucose-deprived control cells, lactate accumulation over a 24-h period was very low (5.9 ± 0.7 μmol/mg of protein). Lactate levels were not increased by incubation with 5 mM glycerol, consistent with the low capacity of these cells for glycerol metabolism. Addition of glucose caused a more than 10-fold increment in lactate concentration (to 75 ± 6 μmol/mg of protein) that was not further altered by concomitant addition of glycerol. In glucose-deprived AdCMV-GIK-treated cells, addition of 5 mM glycerol increased lactate production by more than 2-fold (to 16.7 ± 2 μmol/mg of protein) compared with glucose-deprived control cells. AdCMV-GIK-treated cells incubated with glucose and without glycerol produced an amount of lactate similar to that of control cells (72 ± 5 μmol/mg of protein). In contrast to our findings in control cells, concomitant addition of glycerol and glucose to AdCMV-GIK-treated cells resulted in lower lactate levels than seen with glucose alone (23 ± 2 μmol/mg of protein). This may be explained by the fact that high glycerol 3-P levels may alter the equilibrium of the glycerol phosphate dehydrogenase reaction in favor of dihydroxyacetone phosphate production, thus eliminating a source of NAD⁺ for the glyceraldehyde-3-phosphate dehydrogenase reaction of glycolysis (19).

DISCUSSION

Blood glycerol has been shown to be taken up by skeletal muscle and used as a precursor for intramuscular TAG synthesis in rats (7), reinforcing the notion that functional GIK is present in this tissue (8). However, the interpretation of the in vivo data was hampered by the fact that some of the infused glycerol may have been converted to glucose in the liver prior to its use as a substrate by muscle. In the present study, we have assessed the capacity of cultured human muscle cells to metabolize glycerol and have evaluated the potential rate-limiting role of GIK.

Human muscle cells in culture exhibited very low GIK activity, the specific activity being 4 times lower than that detected in fresh rat gastrocnemius muscle tissue. Consistently, cultured cells did not accumulate significant amounts of glycerol 3-P in response to incubation with glycerol. Incubation with lactate, a putative glyceroneogenic substrate (20), did not raise glycerol 3-P, whereas glucose doubled its concentration. Nevertheless, in cells treated with [U-14C] glycerol, incorporation of radioactivity into both PL and TAG fractions was detected even in the presence of glucose, indicating that these cells are able to metabolize glycerol and use it for lipid synthesis at a low rate. Our data agree with a previous report showing incorporation of [14H] glycerol into PL and neutral lipids in BC3H-1 myocytes and enhancement of these pathways by insulin (21). Nevertheless, in this study, only short term effects of insulin were investigated, and no evidence of glycerol 3-P accumulation was found. In the current study, we show that long term incubation with insulin raises GIK activity and glycerol 3-P accumulation, suggesting a regulatory role of GIK.

To evaluate the precise role of the glycerol phosphorylation step in control of glycerol uptake and metabolism, muscle cells were engineered to overexpress the GIK gene from Escherichia coli. There is a 50% sequence identity between the GIK proteins of E. coli and humans with regions of 100% identity (10). We show that adenovirus-mediated overexpression of GIK in muscle cells unveils a glycerol concentration-dependent increase in glycerol phosphate levels that is not apparent in control cells. This result implies that glycerol transport in muscle cells is very high and that phosphorylation is rate-limiting. This is compatible with the fact that glycerol concentrations are very high in muscle interstitial fluid, suggesting high efflux from TAG hydrolysis and limited reuptake (1). The increased capacity for glycerol phosphorylation in AdCMV-GIK-treated cells was reflected in a large increase in the incorporation of glycerol into cellular lipids as long as fatty acids (oleate) were also provided. This result demonstrates that all of the other enzymes required for lipid esterification are present at high levels in muscle cells. This is in contrast to other cell types with low GIK activity such as pancreatic islet β-cells in which the overexpression of GIK led to a minor increase in incorporation of glycerol into lipids (12).

An intriguing finding of our study is that muscle cells treated with AdCMV-GIK also exhibit an enhanced capacity to metabolize glycerol to glycogen or lactate. This indicates that glycerol 3-P formed from glycerol is transformed into dihydroxyacetone phosphate through the reaction catalyzed by the cytosolic or mitochondrial forms of glycerol-3-P dehydrogenase, both of which are highly expressed in skeletal muscle (22). Dihydroxyacetone phosphate then can enter the glycolytic or gluconeogenic pathway for conversion to lactate or to form glucose moieties that may be incorporated into glycogen, respectively. Importantly, glycerol incorporation into glycogen was doubled in GIK-overexpressing cells in which the rate-limiting enzyme
of this pathway, glycogen synthase, was activated by overexpression of PTG (18). Thus, overexpression of GIK in muscle cells allows glycogen to be utilized in much the same fashion as it is in normal liver cells with the exception that muscle cells do not produce free glucose from gluconeogenesis as liver cells do. It has long been suspected that glycogenesis in muscle is fueled by substrates others than glucose, especially in the postexercise period. Lactate, which accumulates in muscle, is one of the glut4 transporter and low expression of glycogen-metabolizing enzymes (5, 25), although extrapolation to mature human skeletal muscle is inevitably speculative. On the other hand, we show that in cultured muscle cells have a metabolic profile more similar to type I fibers, whereas it is a primary pathway in type II fibers (24). Our data in cultured muscle show that lactate is not used as a glyconeogenic substrate; this may be because cultured muscle cells have a metabolic profile more similar to type I fibers because of the high expression of the GLUT1 transporter and low expression of glycogen-metabolizing enzymes (5, 25), although extrapolation to mature human skeletal muscle is inevitably speculative. On the other hand, we show that in muscle cells with high levels of functional GIK, glycerol may be incorporated into glycogen. Given the fact that freshly isolated muscle samples contain 4 times as much endogenous GIK as the cultured myocytes in this study, glycogen synthase from glyceraldehyde 3-phosphate dehydrogenase in muscle cells is a physiologically relevant pathway for muscle glycogen storage in vivo, particularly in the postexercise period. In this situation, two events would converge. First, glycogen concentration will be high due to mobilization of intramuscular TAG (1, 26). Second, glycogen synthase is maximally activated due to glycogen depletion (27, 28). Further work will be necessary to demonstrate the potential use of glycogen for glycogen synthesis in muscle in vivo.

In summary, we demonstrate that GIK activity is present in cultured human muscle cells. This enzyme is functional, is inducible by insulin, and enables muscle cells to incorporate glyceraldehyde 3-phosphate into lipids. Moreover, overexpression of GIK reveals that this enzyme is the limiting step in muscle glycogen metabolism and that elevated glyceraldehyde 3-phosphate can be diverted toward the glycogeneic-glycolytic pathway leading to glycogen formation and lactate synthesis.

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Effects of Modulation of Glycerol Kinase Expression on Lipid and Carbohydrate Metabolism in Human Muscle Cells
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