The effect of increased expression of glycogen phosphorylase on glucose metabolism in human muscle was examined in primary cultured fibers transduced with recombinant adenovirus AdCMV-MGP encoding muscle glycogen phosphorylase. Increments of 20-fold in total enzyme activity and of 14-fold of the active form of the enzyme were associated with a 30% reduction in basal glycogen levels. Total glycogen synthase activity was doubled in AdCMV-MGP-transduced cells even though the activity ratio was decreased. Incubation with forskolin, which inactivated glycogen synthase and activated glycogen phosphorylase, induced greater net glycogenolysis in engineered cells. In unstimulated fibers, lactate production was three times higher in AdCMV-MGP fibers as compared with controls, despite similar rates of glycogenolysis. In transduced fibers incubated with 2-deoxyglucose, the level of 2-deoxyglucose 6-phosphate was about 8-fold elevated over the control even though hexokinase activity was unmodified in AdCMV-MGP fibers. Overexpression of glycogen phosphorylase also led to enhancement of [U-14C]glucose incorporation into glycogen, lactate, and lipid. Accordingly, determination of lipid cell content revealed that engineered cells were accumulating lipids. Furthermore, 14CO2 formation from [U-14C]glucose was 1.6-fold higher, whereas 14CO2 formation from [6-14C]glucose was unmodified, in AdCMV-MGP fibers. Our data show that in human skeletal muscle cells in culture, the increase in glycogen phosphorylase activity is able to up-regulate glycogen synthase activity indicating the enhancement of glycogen turnover. We suggest that the increase in glycogen phosphorylase and, thereby, in glycogen metabolism, is sufficient to enhance glucose uptake in the muscle cell. Glucose taken up by engineered muscle cells is essentially disposed of through nonoxidative metabolism and converted into lactate and lipid.

Glycogen phosphorylase is the rate-limiting enzyme of glycogen breakdown. In muscle, glycogen serves mainly to provide glucose for energy production during exercise, although it is also consumed in the resting state. During exercise muscle glycogenolysis is triggered by the dual control of contractile activity and epinephrine (1). These stimuli result in the release of Ca2+ and an increase in cyclic AMP, respectively, which in turn lead to the phosphorylation and activation of glycogen phosphorylase by phosphorylase kinase (2).

During exercise, glucose uptake and metabolism are greatly increased in muscle despite low physiological concentrations of insulin. Indeed, it has been shown that insulin is not required to mediate glucose uptake during contractions (3, 4) and that contractile activity augments glucose uptake by muscle even in severely insulin-deficient diabetic rats (5). Furthermore, in exercised muscle, glucose uptake and disposal are enhanced independently of insulin (6). Insulin sensitivity of glucose uptake and glycogen synthesis are increased in exercised muscle, in normal humans and insulin-deficient type I diabetic patients (7, 8). The mechanism by which exercise increases basal and insulin-stimulated muscle glucose uptake remains to be elucidated. The system accounting for such effects appears to be located at a post-receptor level, because exercise does not affect the amount of insulin receptors or insulin-stimulated kinase activity (9, 10). Breakdown of glycogen stores (11–13) or the activation of glycogen synthase (9) have been suggested as possible mediators of this phenomenon. On the other hand, even though these studies are consistent with the fact that glucose uptake is limited by glucose metabolism, other studies suggest that it is glucose transport that limits glucose uptake (14, 15).

We have previously shown that adenoviruses constitute a very efficient vehicle to deliver DNA into nondividing fused C2C12 myotubes (16). In this study, we have used adenoviruses bearing the rabbit muscle glycogen phosphorylase cDNA to increase the expression of the enzyme in human myotubes in culture. This approach has allowed us to evaluate the contribution of phosphorylase to the regulation of glucose metabolism in human muscle fibers and the repercussion of the stimulation of the glycogenolytic process. Our data show that human muscle fibers overexpressing glycogen phosphorylase show a higher capacity for glucose uptake and metabolism through nonoxidative glycolysis and lipid synthesis. These results may be related to the physiological mechanism by which muscle glucose disposal is increased during and after exercise.

MATERIALS AND METHODS

Human Muscle Primary Cultures—Human muscle cultures were initiated from satellite cells of muscle biopsies of patients considered free of muscle disease after all diagnostic studies were reported. Aneural muscle cultures were established in monolayer according to the explant re-expansion technique described by Askanas and Engel in Ref. 17 and modified as in Ref. 18. The cultures were grown in a Dulbecco’s modified Eagle’s medium/M-199 medium (proportion, 3:1; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), 10 μg/ml insulin (Sigma), 20 mM glutamine (Sigma), 25 ng/ml fibroblast growth factor (Collaborative Biomedical Products), 10 ng/ml epidermal growth factor (Collaborative Biomedical Products), and an antibiotic-antimycotic mixture (Life Technologies, Inc.). Immediately after myoblast fusion, cells were rinsed with Hanks’ balanced salt solution (Life Technologies, Inc.), and the medium was replaced by

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a medium devoid of fibroblast growth factor, epidermal growth factor, and glutamine. To perform metabolic studies, except where otherwise stated, cells were switched to a medium devoid of insulin and fetal serum and including 5 mM glucose 2 h before the beginning of the experiment. This condition was considered the basal state. Experiments were performed using muscle cultures established from the muscle biopsies of seven different patients.

Construction of Recombinant Adenovirus AdCMV-MGP and Cell Infection—The construction of AdCMV-MGP, which contains an expression unit consisting of the cytomegalovirus promoter, a 2.56-kilobase fragment of the rabbit muscle glycogen phosphorylase cDNA including the entire coding region (19), and a fragment of the SV40 genome, which includes a polyadenylation signal, has been previously described (20). The recombinant viruses were amplified in 293 cells (21) and viral stocks of 10^7 plaque-forming units/ml were prepared. Guinea pigs were inoculated subcutaneously with 10^7 plaque-forming units of virus per animal. Mice were inoculated intraperitoneally with 10^6 plaque-forming units of virus per animal.

RESULTS

Glycogen Metabolism and Lactate Production—In the basal condition, glycogen content in AdCMV-MGP-transduced cells was 70% lower than in untransduced cells (Fig. 1). When cells were stimulated to mobilize glycogen by incubation with forskolin, glycogen was degraded in transduced cells to undetectable levels upon 120 min of incubation. As expected, in AdCMV-MGP fibers total glycogen phosphorylase activity (measured in the presence of the activator AMP) was clearly higher (20-fold over the control) (Table I), as was phosphorylase a activity (assayed without AMP), which increased 14-fold. Therefore, in transduced cells, the AMP/AMP activity ratio was significantly lower, suggesting that a high proportion of the exogenous glycogen phosphorylase was kept inactive in the basal state (Fig. 2). After incubation of the cells with forskolin, a time-dependent activation of phosphorylase a could be observed in both control and transduced cells (Fig. 2). A 1.3-fold increment in the AMP/AMP activity ratio was obtained in control cells, which was already maximal after 20 min of incubation and had disappeared after 120 min. In contrast, an increment of about 2-fold was detected in AdCMV-MGP cells, which led to activity ratio values (around 0.85) higher than that of control cells (about 2-fold increment) (Table I).

In AdCMV-MGP cells, total glycogen synthase activity (assayed in the presence of 10 mM glucose 6-P) was significantly higher than that of control cells (about 2-fold increment) (Table I). In contrast, the active form (assayed without glucose 6-P) was only slightly increased. Thus, the Glc6P/Glc6P activity ratio was lower in transduced cells (Fig. 3). Treatment of the cells with forskolin induced a time-dependent decrease in the Glc6P/Glc6P activity ratio in control and AdCMV-MGP fibers. The maximal inactivation was reached between 40 and 60 min and was of similar magnitude (about 50%) for both type of

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1. S. Baqué, J. J. Guinovart, and A. M. Gómez-Foix, unpublished results.
2. The abbreviation used is: 6-P, 6-phosphate.
cells. By 120 min, the activity ratio had returned to basal values in control and transduced fibers.

We examined whether glycogenolysis resulted in changes in lactate formation. Although control cells showed a very small accumulation of lactate in the medium during a 120-min incubation, cells overexpressing myophosphorylase demonstrated a time-dependent increase in lactate concentration. As shown in Fig. 4, lactate production (calculated as the difference in lactate concentration) in 2 h was 9.55 μmol/mg protein in transduced cells and only 2.27 μmol/mg protein in control cells. It should be mentioned that during this period of time, cellular glycogen content decreased by 0.21 μmol of glucose/mg of protein in transduced cells, and a similar decrease (0.18 μmol of glucose/mg of protein) was observed in control cells (Fig. 1). Therefore, data suggested that the increase in lactate is larger than can be accounted for net glycogen breakdown, suggesting an increase in glucose metabolism.

Stimulation of the Utilization of 14C-Glucose—The rate of [U-14C]glucose utilization and conversion into glycogen, lactate, CO₂, or lipids was determined in cells overexpressing glycogen phosphorylase (Table II). AdCMV-MGP fibers exhibited a marked elevation (1.6-fold increment) in the incorporation of [U-14C]glucose into glycogen. The increase in labeled glycogen, which is not associated with net increase in glycogen content, may be due to an elevation of glycogen turnover. The conversion of [U-14C]glucose into lactate was also enhanced in transduced cells overexpressing glycogen phosphorylase. After 120 min of incubation, the incorporation of [U-14C]glucose into lactate was 4.5-fold higher in transduced cells than in control cells. Incorporation of [U-14C]glucose into total lipids was also analyzed. Increases of about 4.5-fold in the percentage of 14C present in total lipid were observed in transduced myofibers compared with controls. When the distribution of radioactivity between the glycerol moiety and the fatty acid pool was determined, we found that as previously shown (30), in muscle cells most of the radioactivity is present in the glycerol moiety (95%) rather than in the fatty acid fraction. The incorporation of 14C into both fractions was higher in AdCMV-MGP muscle cells with

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**Table I**

| Enzyme activities | Control | AdCMV-MGP |
|-------------------|---------|-----------|
| Total GP (+AMP)   | 70.63 ± 5.75 | 1422.6 ± 117.9 |
| Active GP (-AMP)  | 48.96 ± 3.31  | 714.58 ± 32.4 |
| Total GS (+G6P)   | 2.90 ± 0.08  | 5.54 ± 0.12 |
| Active GS (-G6P)  | 0.085 ± 0.014 | 0.773 ± 0.016 |
| Hexokinase        | 3.31 ± 7.14  | 117.9 ± 142.6 |

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**Fig. 3.** Time-dependent effect of forskolin on glycogen synthase activity ratio. Noninfected (squares) or AdCMV-MGP-transduced fibers (circles) were incubated in the absence (open symbols) or the presence (closed symbols) of 100 μM forskolin for the indicated times. Glycogen synthase (GS) activity was measured as described under "Materials and Methods." The results are the means ± S.E. of four independent experiments performed in duplicate. G6P, glucose 6-P.

**Fig. 4.** Time course of lactate production in human muscle fibers in culture. Human fibers nontransduced (□) or transduced with AdCMV-MGP (○) viruses preincubated as described under "Materials and Methods" were incubated with 10 mM glucose. Lactate accumulated in the medium was measured at the times indicated. The results are the means ± S.E. of four independent experiments performed in triplicate. prot., protein.
similar increases (about 3-fold) in labeling of each fraction.

Examination of the $^{14}$CO$_2$ production revealed that in cells that had been exposed to AdCMV-MGP viruses, $^{14}$CO$_2$ release was elevated 1.6-fold over the controls. $^{14}$CO$_2$ production from [U-$^{14}$C]glucose mainly reflects CO$_2$ derived from the decarboxylation of glucose in the pentose phosphate pathway and in the pyruvate dehydrogenase step in the pathway of fatty acid synthesis. Because lipid synthesis was stimulated in these cells, we used [6-$^{14}$C]glucose to determine whether the overexpression of myophosphorylase also stimulated glucose oxidation. We found that there was no difference in the $^{14}$CO$_2$ production from [6-$^{14}$C]glucose in AdCMV-MGP-transduced myofibers compared with control cells. Therefore, the observed increase in $^{14}$CO$_2$ released from [U-$^{14}$C]glucose was essentially due to lipid synthesis.

Glucose Uptake and Phosphorylation—The effect of phosphorylase dosage on the uptake and phosphorylation of the non-metabolizable glucose analogue 2-deoxyglucose was studied (Table III). 2-Deoxyglucose is transported into the muscle cell and at low doses almost entirely phosphorylated. In AdCMV-MGP-transduced cells incubated with 2-deoxyglucose, hexose 6-P intracellular concentration was enhanced by about 7.7-fold after 60 min of incubation with 10 mM 2-deoxyglucose, meaning that uptake of glucose was activated. Glucose 6-P levels in cells preincubated for 2 h in the absence of 2-deoxyglucose or glucose were slightly higher in engineered cells (Table III). Higher glucose 6-P levels were observed in cells overexpressing glycogen phosphorylase (74.3 ± 4.1 nmol/g protein) with respect to control cells (33.2 ± 2.7 nmol/g protein) when fibers were continuously incubated in the presence of 30 mM glucose and 1 μM insulin. The increases in the hexose 6-P levels cannot be attributed to the rise in hexokinase activity, because this activity was unmodified in cells overexpressing phosphorylase (Table I).

Effect on Lipid Accumulation—Because incorporation of $^{14}$C-glucose into lipid suggested that muscle lipogenesis was stimulated in cells overexpressing glycogen phosphorylase, it was determined whether these cells presented accumulation of lipids. As shown in Fig. 5, AdCMV-MGP-transduced cells showed intense staining for lipids, whereas control cells were virtually negative. Quantification of total lipid content by gravimetry showed that engineered cells had 516 ± 21 μg of lipid/mg of protein with respect to 292 ± 17 μg of lipid/mg of protein in controls.

**DISCUSSION**

A remarkable consequence of the overexpression of phosphorylase is an enhancement of glucose uptake and consumption by the muscle cell, probably secondary to increased turnover and utilization of glycogen stores. Consistent with this interpretation, we found that engineered cells exhibited an enhancement in the production of lactate that could not be explained solely by net depletion of glycogen. Although depletion of glycogen was similar in control and AdCMV-MGP cells, the rate of net lactate production was much higher in engineered cells, as was the incorporation of radioactivity from $^{14}$C-glucose into lactate. Because the incorporation of $^{14}$C-glucose into glycogen was also increased in engineered cells, one possible explanation for the increase in lactate production is that glycogen turnover is stimulated, leading to an increase in glucose conversion into glycogen and its subsequent degradation to lactate. Moreover, AdCMV-MGP-transduced cells showed an enhanced accumulation of the phosphorylated metabolite of 2-deoxyglucose, a glucose analog that is not metabolized beyond its 6-phosphorylated form. The increase in 2-deoxyglucose 6-P concentration found in engineered cells seems to reflect their elevated capacity to transport glucose, because hexokinase activity is unchanged by overexpression of phosphorylase. Our results may be relevant to the increase in glucose uptake and metabolism observed during exercise. It has been long hypothesized that exercise stimulation of glucose transport is related to the lowering of muscle glycogen stores. In vivo, exercise activates glycogen phosphorylase by a dual control of epinephrine and contractile activity, and both stimulating events have been associated to the enhancement of glucose uptake. In isolated

**TABLE I**

| Glucose compounds         | Control | AdCMV-MGP |
|---------------------------|---------|-----------|
| Glycogen                  | 100     | 169 ± 2   |
| Lactate                   | 100     | 455 ± 30  |
| $^{14}$C(U)-CO$_2$         | 100     | 162 ± 6   |
| $^{14}$C(6)-CO$_2$         | 100     | 99 ± 0.9  |
| Total lipid               | 100     | 447 ± 20  |
| Total lipid fatty acid    | 100     | 341 ± 39  |
| Total lipid glycerol      | 100     | 311 ± 27  |

**TABLE II**

Radioactivity in glycogen, lactate, CO$_2$, and lipid in cultured human muscle fibers incubated with [U-$^{14}$C]glucose or [6-$^{14}$C]glucose

Uninfected fibers (Control) or AdCMV-MGP-transduced fibers (AdCMV-MGP) were incubated for 2 h with 10 mM [U-$^{14}$C]glucose for determination of glycogen, lactate, and [U-$^{14}$C]-CO$_2$, or with 10 mM [6-$^{14}$C]glucose for determination of [6-$^{14}$C](6)-CO$_2$, or 20 mM [U-$^{14}$C]glucose for lipids. The results are expressed as the percentage of control. The data are the means ± S.E. for at least five independent experiments performed in triplicate.

**TABLE III**

2-Deoxyglucose uptake in human cultured skeletal muscle

Uninfected fibers (Control) or AdCMV-MGP-transduced fibers (AdCMV-MGP) were incubated for 1 h with 0, 10, or 30 mM 2-deoxyglucose (2DG). Following incubation, hexose 6-P was measured in cell extracts as described under "Materials and Methods." The results are the means ± S.E. for three independent experiments performed in triplicate.

**Fig. 5. Histochemical staining of lipid content.** Representative phase contrast micrographs (×100) of Oil Red O-stained cultured human fibers untransduced (A) or transduced with AdCMV-MGP (B) 7 days after infection.
muscles it has been observed that a bout of glycogen-depleting exercise increases basal glucose transport (4, 31). In addition, in rats injected with epinephrine, which activates phosphorylase and reduces glycogen stores independent of exercise, basal glucose transport activity is increased (13). We demonstrate that cultured human muscle fibers with higher levels of glycogen phosphorylase activity show an enhanced capacity for glucose uptake and consumption, despite having a relatively unaltered rate of glycogen depletion and unmodified hexokinase activity.

Controversy exists regarding whether it is glucose transport or its intracellular metabolism that limits glucose utilization by muscle tissue (14, 32). Our data provide evidence that it is the intracellular glucose utilization that limits the rate of glucose uptake. We show that in AdCMV-MGP-transduced cells, along with the increase in glucose uptake, there is a stimulation of [U-14C]glucose conversion into lactate, lipids, and glycogen. We propose that the influx of glucose triggered by the overexpression of phosphorylase increases glucose disposal to lactate and lipid. Accordingly, we found that the concentration of the intermediate metabolite glucose 6-P is increased in engineered cells (as described under “Results”). The reverse situation has been observed in muscle fibers from myophosphorylase-deficient patients (McArdle’s disease) in which the levels of intermediate glycolytic metabolites such as glucose 6-P are depleted (33). Moreover, our results suggest that the moderate increase in [14C]glucose is due to the pentose pathway or the pyruvate dehydrogenase step rather than to the oxidation of glucose in the Krebs cycle. Therefore, the glucose taken up by AdCMV-MGP muscle cells appears to be essentially consumed through nonoxidative metabolism.

Our data also show that overexpression of glycogen phosphorylase induces an increase in total glycogen synthase activity even though glycogen synthase mRNA levels appear to be unmodified (data not shown). Even though total glycogen synthase activity is increased, there is only a small increase in the level of the active form, and thus the activity ratio is decreased. These results might be related to previous observations showing that exercise increases the total activity of both glycogen synthase and glycogen phosphorylase (34, 35), conferring to the muscle cells enhanced capacity for glycogen depletion and re-synthesis. Additionally, Westergaard and colleagues (36) demonstrated in athletes an elevation of total glycogen synthase activity together with a decrease in the activity ratio, which is accompanied by no difference in immunoreactive glycogen synthase. As in this study, it is suggested that post-translational modifications of the enzyme and not regulation of gene expression seem to account for modulation of glycogen synthase activity in muscle. The fact that an increase in glycogen phosphorylase activity results in an increase in the activity of glycogen synthase suggests the presence of a local control of glycogen turnover. It seems that a compensatory mechanism exists that tends to equilibrate the rates of glycogenolysis and glycogenesis to maintain glycogen turnover and net glycogen content. The simultaneous existence of both reactions or glycolytic cycling has been clearly demonstrated in liver (37). Furthermore, in liver, glycogen turnover is greater in the fed state than in the fasted state (38), and thus it has been suggested that glycogen concentration may exert a regulatory effect on glycogen turnover. We found that in AdCMV-MGP-treated muscle cells, there is a compensatory mechanism that up-regulates glycogen synthase activity following the increase in phosphorylase, despite a moderate decrease in glycogen content. Therefore, our data suggest the involvement of local undetermined regulatory factors.

It is concluded that in human skeletal muscle cells, the increase in glycogen phosphorylase activity is sufficient to up-regulate glycogen metabolism and to drive the uptake of glucose. Therefore, increased intracellular metabolism of glucose may be the primary event in the induction of a higher capacity to take up glucose by skeletal muscle. Our finding is related to the exercise-induced enhancement in muscle glucose transport. Additionally, we show that the increase in glucose utilization is associated with increased production of lactate and accumulation of lipid. In summary, it is shown that muscle cells respond to the increase in the glycolytic capacity by increasing glucose uptake and consumption.

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