Original Articles

Control of Glycogen Synthesis by Glucose, Glycogen, and Insulin in Cultured Human Muscle Cells

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A key feature of type 2 diabetes is impairment in the stimulation of glycogen synthesis in skeletal muscle by insulin. Glycogen synthesis and the activity of the enzyme glycogen synthase (GS) have been studied in human myoblasts in culture under a variety of experimental conditions. Incubation in the absence of glucose for up to 6 h caused an ~50% decrease in glycogen content, which was associated with a small decrease in the fractional activity of GS. Subsequent reincubation with physiological concentrations of glucose led to a dramatic increase in the rate of glycogen synthesis and in the fractional activity of GS, an effect which was both time- and glucose concentration–dependent and essentially additive with the effects of insulin. This effect was seen only after glycogen depletion. Inhibitors of signaling pathways involved in the stimulation of glycogen synthesis by insulin were without significant effect on the stimulatory action of glucose. These results indicate that at least two distinct mechanisms exist to stimulate glycogen synthesis in human muscle: one acting in response to insulin and the other acting in response to glucose after glycogen depletion, such as that which results from exercise or starvation. *Diabetes* 50: 720–726, 2001

Glycogen synthesis in skeletal muscle is under hormonal control, with a principal regulatory role being taken by insulin that acutely promotes glycogen synthesis from glucose by stimulating glucose uptake and by activating the key enzyme glycogen synthase (GS) (1). Stimulation of GS by insulin primarily involves net dephosphorylation of three specific serine residues, collectively termed sites 3 (2). The kinase principally responsible for phosphorylation of sites 3 is GS kinase (GSK)-3, whereas dephosphorylation is mediated by a glycogen-bound form of protein phosphatase (PP)-1 (3,4). There is significant evidence that insulin activates GS primarily via inhibition of GSK-3 (5,6). The phosphorylation state of site 2 is also implicated as a possible regulatory factor in the activity of GS (7). Both PP1 and PP2A can dephosphorylate sites 2 and 3 in vitro (2), and there is evidence for the activation of $PP1_G$ by insulin in muscle (4) and both PP1 and PP2C in liver (8), indicating that regulation of GS by insulin is multifactorial. However, there is evidence that the impaired activation of GS by insulin in type 2 diabetes may be associated with increased levels of GSK-3 expression in muscle (9).

Glycogen synthesis can also be stimulated in an insulin-independent manner (after exercise), a process associated with glycogen depletion (10). Furthermore, as early as 1965, a reciprocal relationship between glycogen content and glycogen synthase activity was reported in muscle (11).

Previously, we have used human muscle cells in culture to study the control of glycogen synthesis by insulin and growth factors (12,13). In the current study, we examined the effects of glycogen depletion on the rate of glycogen synthesis and the activity of GS in human myoblasts. The effect of subsequent glucose readdition was also studied, and the mechanism leading to stimulation by glucose of GS was analyzed and compared with that for insulin.

RESEARCH DESIGN AND METHODS

Materials. All tissue culture trays were from Costar (Cambridge, MA). Culture media, penicillin/streptomycin, and trypsin-EDTA were from Gibco-BRL (Paisley, U.K.). Chick embryo extract was obtained from ICN (Costa Mesa, CA). D-[U-^{14}C]glucose (300 mCi/mmol), uridine diphospho-D-[6-^{3}H]glucose (814 GBq/mmol), and 2-deoxy-D-[1-^{3}H]glucose (362 GBq/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). The GSK-3 substrate phospho-eIF2B peptide [RRAAEELDSRAGS(p)PQL] (14) was received from Prof. C.G. Proud (University of Dundee, Dundee, U.K.). Wortmannin and rapamycin were from Sigma (Poole, U.K.), and PD098059 was from New England Biolabs (Beverly, MA). Actrapid insulin was from Novo Nordisk (Copenhagen, Denmark). Anti-GSK-3α and GSK-3β phosphospecific antibodies were from New England Biolabs.

Cell culture. Human myoblasts were grown from needle biopsy samples taken from the gastrocnemius muscle of healthy subjects with no family history of type 2 diabetes and with normal glucose tolerance and normal insulin sensitivity, as assessed using the short insulin tolerance test (13). Myoblasts were maintained in growth medium consisting of Ham's F-10 nutrient mixture containing 20% fetal calf serum (FCS), 1% chick embryo extract, 100 U/ml penicillin, and 100 μg/ml streptomycin. All experiments were performed using cells between the 5th and 15th passage at a confluence >80%. Before hormone treatment, cells were incubated for at least 2 h in serum-free medium.

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DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Glu$^-$, glucose free; G6P, glucose-6-phosphate; GS, glycogen synthase; GSK, GS kinase; PBS, phosphate-buffered saline; PP, protein phosphatase.

Cellular glycogen content determination. Total cellular glycogen content was assessed by modification of a previous method (15). After treatment, cells were washed rapidly in ice-cold phosphate-buffered saline (PBS) and scraped into 100 μl of 0.2 mol/l sodium acetate, pH 4.8. Extracts were briefly sonicated using a Soniprep 150, before the addition of 250 mU amyloglucosidase per sample. Samples were incubated for 2 h at 40°C and vortexed regularly to avoid sedimentation. The sample (10 μl) was incubated with an assay cocktail (0.1 mol/l Tris-HCl [pH 8.0], 0.3 mmol/l ATP, 6 mmol/l $MgCl_2$, 5 mmol/l dithiothreitol, 60 μmol/l $NADP^+$, 2.5 U/ml hexokinase, and 1 μg/ml G6P-dehydrogenase) for 30 min at room temperature. Changes in fluorescence, as a result of NADPH production, were determined using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Reaction blanks were determined as the fluorescence of samples before enzymatic treatment with amyloglucosidase.

Measurement of glycogen synthesis. Glycogen synthesis was determined as ^{14}C-glucose incorporation into glycogen over a 1-h period, as previously described (13). Results were expressed as picomoles of glucose incorporated into glycogen per minute per milligram of cell protein.

Estimation of glucose uptake. Glucose uptake was determined as the rate of 2-deoxy-D-[6-^{3}H]glucose uptake, using a modification of a previous method (16). Cells were maintained in the absence of serum for 2 h before the replacement of media with glucose-free (Glu^-) Dulbecco's modified Eagle's medium (DMEM) for 15 min at 37°C. The rate of 2-deoxy-D-[6-^{3}H]glucose uptake was determined during a 5-min incubation with 10 μmol/l 2-deoxy-D-[6-^{3}H]glucose (specific activity 400 dpm/pmol). Reaction blanks were determined as the rate of 2-deoxy-D-[6-^{3}H]glucose uptake in the presence of 0.1 mmol/l cytochalasin B.

After incubation, cells were washed with ice-cold PBS several times and solubilized in 0.05% SDS for 30 min at room temperature. Protein content of samples was assayed using Coomassie protein assay reagent, and uptake of 2-deoxy-D-[6-^{3}H]glucose was determined by liquid scintillation counting.

Assay of glycogen synthase. Following the indicated treatments, cells were rapidly washed three times with ice-cold PBS and collected, by scraping, into GS extraction buffer (10 mmol/l Tris-HCl [pH 7.8], 150 mmol/l KF, 15 mmol/l EDTA, 60 mmol/l sucrose, 1 mmol/l 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mmol/l benzamidine, and 1 mmol/l phenylmethylsulfonyl fluoride). Cells were then disrupted by briefly sonicating using a Soniprep 150. Glycogen synthase activity was determined in whole lysates as the incorporation of ^{3}H-glucose from uridine-5′-diphosphate [U-^{3}H] glucose into glycogen, as previously described (17). Samples were incubated with reaction cocktail (50 mmol/l Tris-HCl [pH 7.8], 20 mmol/l EDTA, 25 mmol/l KF, 1% glycogen, and 0.4 mmol/l UDP-[^{3}H]glucose [specific activity 3,000 dpm/nmol]), containing either 0.1 mmol/l (active) or 10 mmol/l (total) glucose-6-phosphate for 30 min at 30°C. Results were expressed as fractional activities (active/total). This assay has been optimized to detect the activity changes resulting from dephosphorylation of glycogen synthase (17).

Determination of GSK-3 activity. Following treatment, cells were washed three times with ice-cold PBS and excess liquid was removed. Cells were then scraped into kinase extraction buffer (100 mmol/l Tris-HCl [pH 7.4] containing 100 mmol/l KCl, 2 mmol/l EDTA, 25 mmol/l KF, 0.1% [vol/vol] Triton X-100, 1 mmol/l benzamidine, 0.1 mmol/l Na_3VO_4, 1 μg/ml pepstatin, 1 μg/ml antipain, and 1 μg/ml leupeptin), transferred to 1.5 ml Eppendorf tubes, and immediately frozen in liquid nitrogen. GSK-3 activity was determined by a modification of the method reported by Ryves et al. (18), in the presence or absence of 50 mmol/l LiCl (an allosteric inhibitor of GSK-3). Samples (15 μg) were incubated with reaction mixture (50 mmol/l Tris-HCl [pH 7.5] containing 0.1 mmol/l EGTA, 100 mmol/l Mg-Acetate, 5 μmol/l protein kinase inhibitor, 100 μmol/l [γ-^{32}P]ATP [~600 cpm/pmol], and 0.2 mg/ml phospho-eIF2B peptide substrate) (14) for 15 min at 30°C. After incubation, samples containing the radiolabelled peptide product were spotted onto 3 cm^2 Whatman P81 phosphocellulose paper squares. After washing in 175 mmol/l phosphoric acid with four changes, the papers were dried, and phosphate incorporation was determined by liquid scintillation counting. Phosphate incorporation into peptide substrate in the presence of lithium was equivalent to that observed using control dephosphopeptide. GSK-3 activity was regarded as the difference between values in the absence and presence of lithium. Enzyme activity was defined as that which catalyzes the incorporation of 1 nmol of phosphate into peptide substrate in 1 min.

Western blotting. Samples were fractionated on 10% gels by SDS-PAGE. After separation, proteins were transferred onto polyvinylidine fluoride membrane and probed with anti–GSK-3α and GSK-3β phosphospecific antibodies (1:1,000). Anti-rabbit peroxidase conjugate (1:2,000) was used as secondary antibody, and immunoreactive proteins were determined using enhanced chemiluminescence. Membranes were stained with protein-reactive copper stain (0.05% [wt/vol] copper phthalocyanine 3,4′,4′,4′-tetrasulphonic acid in 12 mmol/l HCl) to allow molecular weight estimation of immunoreactive proteins, as compared with those of proteins of known molecular weight.

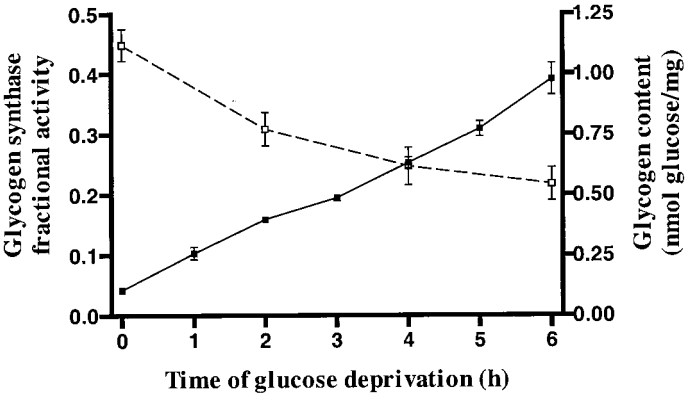

FIG. 1. Total glycogen content and GS activity in myoblasts cultured in the absence of glucose; effect of glucose readdition. Myoblasts were maintained for up to 6 h in DMEM Glu^-. At the times indicated, total glycogen content of the cultures was determined (– – – –, right *y*-axis). In the absence of glucose readdition, the GS activity was less, at every time point, then what was observed in nonstarved cultures. In separate cultures, DMEM Glu^- was replaced with media containing 6.1 mmol/l glucose (Ham's F-10) for 15 min before extraction. The fractional activity of glycogen synthase was determined in these extracts (——, left *y*-axis). Results represent the mean ± SE (n = 7) in at least three subjects.

RESULTS

Effects of glucose withdrawal on glycogen content and GS activity. Human myoblasts were routinely maintained in Ham's F-10 medium, which contains physiological levels of glucose (6.1 mmol/l). Under these culture conditions, the cells accumulate significant amounts of glycogen. To deplete these stores of glycogen, cells were deprived of glucose for increasing periods of time in a glucose-deficient medium of similar composition to Ham's F-10, i.e., DMEM Glu^- (Ham's F-10 lacking glucose is not commercially available) (Fig. 1). A decrease in cellular glycogen content of ~50% was observed in myoblasts maintained in DMEM Glu^- for 6 h, as compared with control cells maintained in the presence of glucose (glycogen content of 0.54 ± 0.07 μmol glucose/mg protein in glucose-deprived myoblasts vs. 1.12 ± 0.06 μmol glucose/mg protein in control myoblasts). This decrease was time-dependent, with a significant effect being observed after 2 h. During this time period, the fractional activity of GS also fell slightly, decreasing after 5 h of glucose starvation from 0.032 ± 0.008 in control cells to 0.014 ± 0.005 (n = 7, from three different subjects). However, if after incubation in the absence of glucose for 6 h Ham's F-10 media (containing 6.1 mmol/l glucose) was returned to the cells for 15 min, a dramatic increase in the fractional activity of GS to 0.390 ± 0.03 was observed (Fig. 1). To confirm that the stimulatory effect is because of glucose restoration, after incubation in DMEM Glu^- for 6 h, cells were exposed to DMEM containing 5 mmol/l glucose. This again caused a dramatic activation of GS to a fractional activity of 0.064 ± 0.01. Supplementation of DMEM with pyruvate (1 mmol/l) and glucose further increased this value to 0.186 ± 0.02 (pyruvate alone had no effect), whereas DMEM, which contains 5.5 mmol/l glucose and 1 mmol/l pyruvate, activated GS to 0.345 ± 0.033. To confirm that alterations in GS activity were a result of previous glucose

withdrawal, cells were incubated with DMEM supplemented with 5.5 mmol/l glucose for 5 h before treatment with Ham's F-10. No increase in the GS activity was observed after Ham's F-10 readmission (data not shown). These data indicate that the stimulation of GS is predominantly due to the action of glucose and requires previous glycogen depletion because of glucose removal, but other components of the different media may modulate the magnitude of the response. In subsequent experiments, Ham's F-10 was used for glucose replenishment, except in Fig. 2*C*, where the effects of varying amounts of glucose were examined, and in Fig. 5, where DMEM Glu^- was supplemented by glucose at t = 0, because of the necessity of adding glucose after preincubation with inhibitors.

The increase in GS fractional activity in response to glucose was dependent on the duration of previous glucose deprivation and, hence, inversely related to the glycogen content of the cells. In the absence of glycogen depletion, no significant activation of GS by glucose was observed (Fig. 1). Total GS activity remained essentially unchanged in all conditions, indicating that alteration in the expression of the GS polypeptide was not involved (not shown).

Time and concentration dependence of GS activation by glucose in glycogen-depleted myoblasts. Myoblasts were incubated in DMEM Glu^- for 5 h before treatment with glucose-containing media for increasing periods of time. Glucose treatment (6.1 mmol/l) caused a rapid time-dependent increase in the fractional activity of GS, with stimulation being observed within 2 min and reaching a maximum fractional activity of ~0.3 after 10–15 min (Fig. 2*A*). After 30 min of glucose re-administration, the fractional activity of GS started to decrease, reaching ~0.1 after 4 h, but remaining constant thereafter up to 8 h (Fig. 2*B*). During the same period, the glycogen content of the cells increased, reaching a value ~80% of that in control cells. The stimulation of GS was dependent on the concentration of glucose added, with significant stimulatory effects being observed in response to 1.5 mmol/l (Fig. 2*C*).

Combined effect of insulin and glucose on GS activity and glycogen synthesis in glycogen-depleted myoblasts. The combined effects of glucose readdition and insulin on cells cultured in the absence of glucose was then examined. Treatment of cells with 100 nmol/l insulin for 15 min after preincubation in glucose-containing media for 5 h led to an ~1.6-fold increase in the fractional activity of GS from 0.040 ± 0.010 to 0.071 ± 0.020 (Fig. 3*A*). Similarly, GS activity was increased by insulin ~1.7-fold from 0.214 ± 0.021 to 0.337 ± 0.037 in myoblasts that had been glucose-depleted for 5 h and that had glucose re-administered during the 15 min of insulin treatment; this finding indicates that the effects of glucose and insulin are additive and act by different mechanisms. In the absence of readministration of glucose, stimulatory effects of insulin on GS activity were minimal after glycogen depletion (data not shown).

Treatment of control cultures with 100 nmol/l insulin for 1 h led to an ~1.9-fold increase in the rate of glycogen synthesis from 134.7 ± 23.45 to 259.8 ± 31.11 pmol · min^{-1} · mg^{-1} protein (Fig. 3*B*). The rate of glycogen synthesis after glucose re-administation for 1 h to cells preincubated in DMEM Glu^- for 5 h was increased dramatically to 944.8 ± 63.79 pmol · min^{-1} · mg^{-1}. This value further increased to 1,493.8 ± 110.47 pmol · min^{-1} · mg^{-1} when insulin was additionally present during the 1 h of glucose refeeding, representing a 1.6-fold stimulation by insulin.

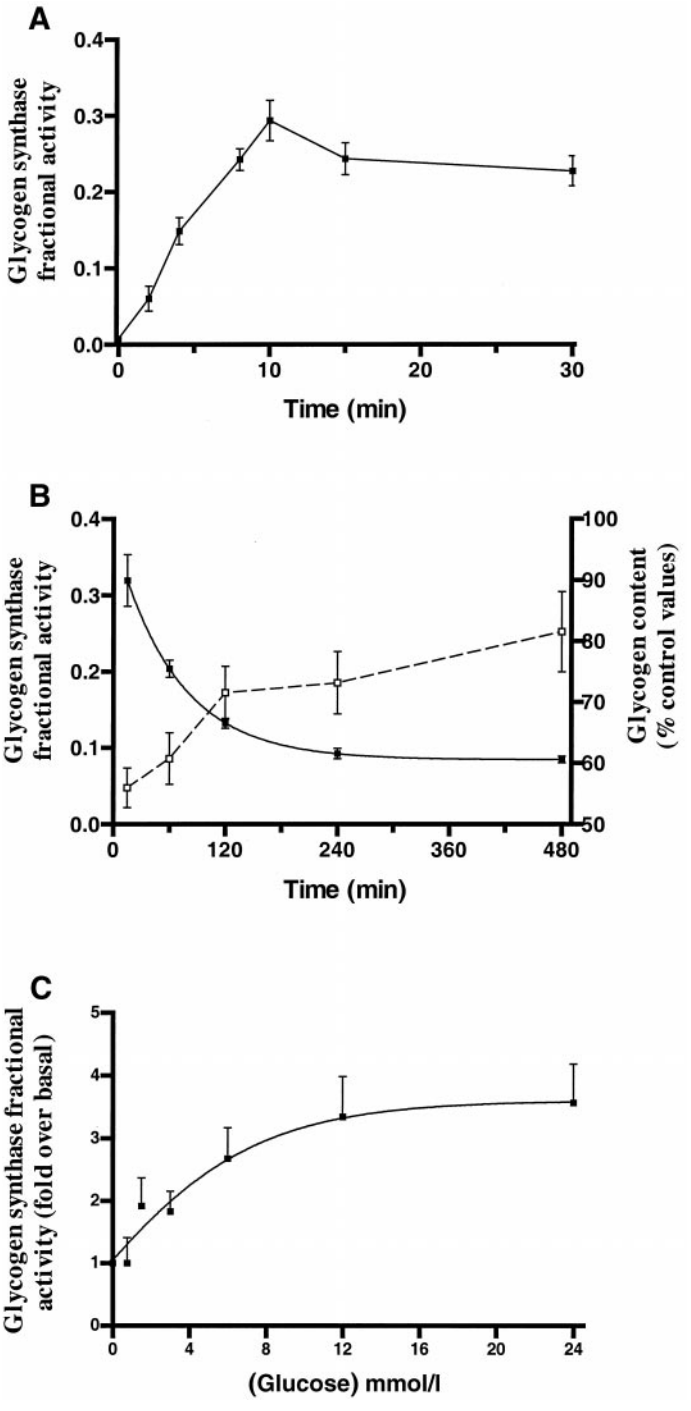

FIG. 2. Concentration- and time-dependent effects of glucose on GS activity in cells cultured in the absence of glucose. Myoblasts were incubated in DMEM Glu^- growth media for 5 h before replacement of media with Ham's F-10 (containing 6.1 mmol/l glucose) for the times indicated (*A* and *B*). Total cellular glycogen content was also determined in cells after chronic refeeding (*B*) (– – – –, right axis). Alternatively, myoblasts were incubated in DMEM Glu^- growth media for 5 h before replacement of media with DMEM Glu^- supplemented with different concentrations of glucose for 15 min (*C*). Extracts were prepared and the fractional activity of glycogen synthase determined. The resulting fractional activities represent the mean ± SE (n = 6) in at least three subjects.

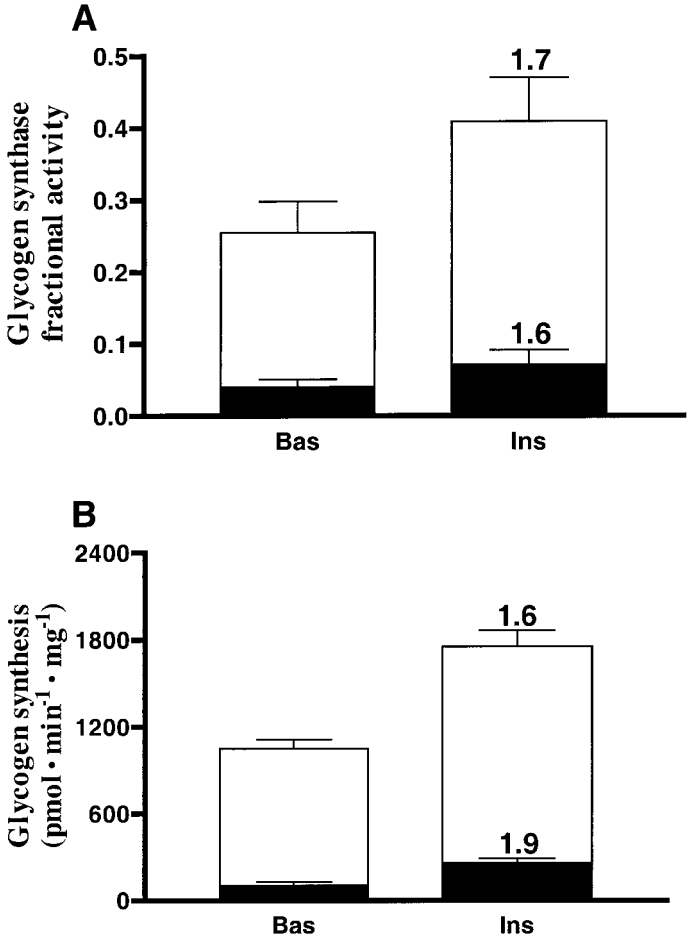

FIG. 3. Combined effects of insulin and glucose on GS activity and glycogen synthesis in cells cultured in the absence or presence of glucose. Cells were incubated in DMEM Glu$^-$ (□) or Ham's F-10 (6.1 mmol/l glucose) (■) for 5 h. Media were then replaced with Ham's F-10 containing 100 nmol/l insulin (Ins) for 15 min or not replaced (Bas) for 15 min and GS activity was determined (*A*); or media were replaced for 1 h and the rate of glycogen synthesis was determined (*B*). Results represent the mean ± SE (n = 6) in at least three subjects. The insulin-mediated fold-activation over basal values is also indicated.

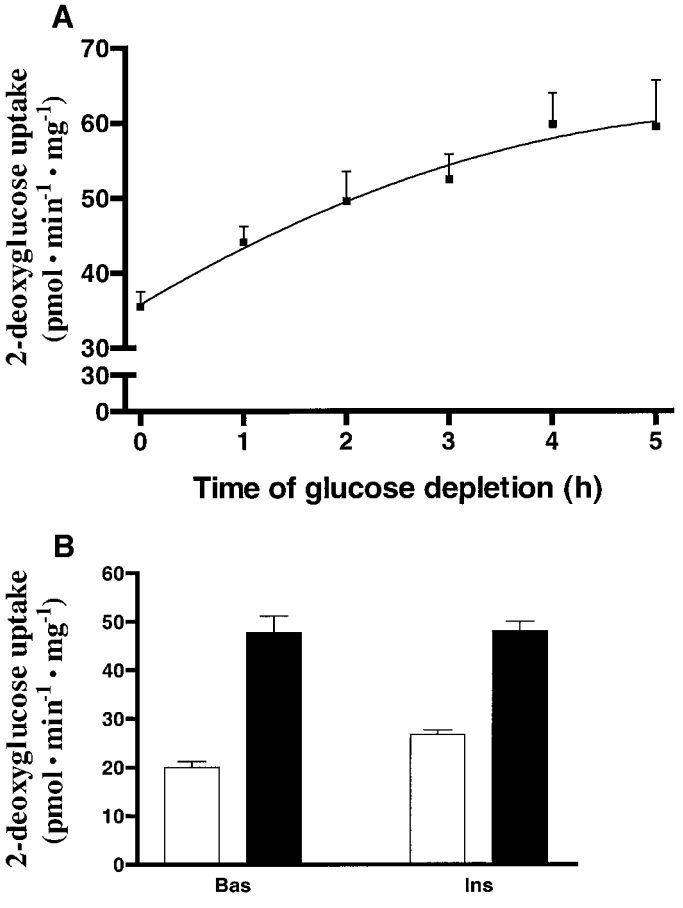

FIG. 4. 2-Deoxyglucose uptake in cells preincubated in the absence of glucose. Cells were maintained in DMEM Glu$^-$ growth media for up to 5 h. At the times indicated, the rate of glucose uptake was determined (*A*). Alternatively, cells were maintained in DMEM Glu$^-$ (□) or Ham's F-10 (6.1 mmol/l glucose) (■) for 5 h before the addition of 100 nmol/l insulin (Ins) or not adding insulin (Bas) for 15 min and the rate of glucose uptake determined (*B*). Results represent the mean ± SE (n = 8) in three subjects.

Effect of glucose deprivation on 2-deoxyglucose uptake in human myoblasts. An increase in the rate of 2-deoxyglucose uptake was observed in myoblasts deprived of glucose for increasing time, reaching 1.7-fold after 5 h (Fig. 4*A*). The most significant increase in the rate of 2-deoxyglucose uptake was observed after the first 2 h in DMEM Glu$^-$: 35.45 ± 1.99 pmol · min^{-1} · mg $^{-1}$ in cells maintained in the presence of glucose and 49.58 ± 3.93 pmol · min^{-1} · mg $^{-1}$ in cells deprived of glucose for 2 h.

The effect of insulin treatment on 2-deoxyglucose uptake was examined in control and glucose-starved myoblasts (Fig. 4*B*). Incubation of control cells with 100 nmol/l insulin for 15 min led to a modest 1.3-fold increase in the rate of 2-deoxyglucose uptake. Insulin (100 nmol/l) treatment failed to further increase 2-deoxyglucose uptake above the increased basal rate observed in glucose-starved myoblasts.

Mechanisms involved in activation of GS by glucose. To investigate the mechanisms by which glucose activates GS, selective inhibitors of signaling pathways known to be stimulated by insulin were used (Fig. 5). DMEM Glu$^-$ supplemented with 5.5 mmol/l glucose was added for 15 min to myoblasts previously depleted of glucose for 5 h, resulting in an ~4.2-fold increase in GS activity ratio. Preincubation of cells with rapamycin, which selectively

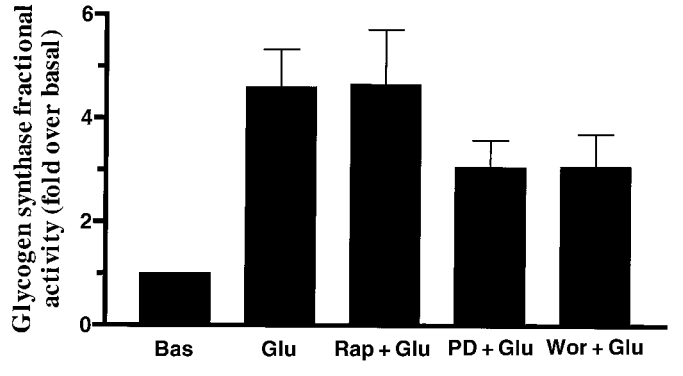

FIG. 5. Effect of selective inhibitors on activation of GS by glucose. Cells were maintained in DMEM Glu$^-$ for 5 h before further incubation for 15 min in DMEM Glu$^-$ (Bas) or DMEM growth media supplemented with 5.5 mmol/l glucose. Alternatively, cells were treated with 100 nmol/l rapamycin (Rap) for 15 min , 50 μmol/l PD98059 (PD) for 1 h, or 100 nmol/l wortmannin (Wor) for 15 min before the addition of glucose (Glu). Extracts were prepared and the activity of GS determined. Results are expressed as the fold-activity over basal (Bas) and represent the mean ± SE (n = 3) in three subjects.

Time of treatment (min)	GSK-3 activity (% of basal)	
	Insulin [10^{-7}]	Glucose [6.1 mmol/l]
0	100	100
2	72.4 (± 11.6)	104.6 (± 5.6)
5	69.5 (± 7.4)*	123.0 (± 10.1)
15	66.8 (± 3.0)*	97.5 (± 7.6)

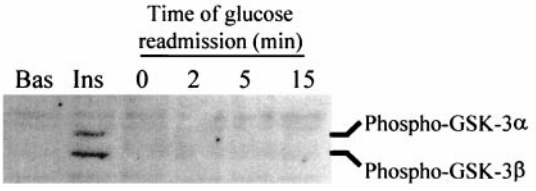

FIG. 6. Effects of insulin and glucose on GSK-3 activity and phosphorylation. Myoblasts were incubated in DMEM Glu$^-$ growth media for 5 h before replacement of media with Ham's F-10 (containing 6.1 mmol/l glucose) for the times indicated. Alternatively, cells were incubated in Ham's F-10 for 5 h before the addition of insulin (100 nmol/l) for the times indicated. Extracts were prepared and GSK-3 activity determined. Data are % of basal activity and mean ± SE (n = 6) in three subjects. Serine phosphorylation of GSK-3α and GSK-3β was probed using phosphospecific antibodies in extracts from samples treated with 100 nmol/l insulin (Ins) or without 100 nmol/l insulin (Bas) for 15 min, or alternatively in myoblasts deprived of glucose for 5 h before readmission of Ham's F-10 for the times indicated. *P < 0.05 compared with basal values.

inhibits the activation of p70^{s6k}, failed to inhibit this stimulatory effect of glucose on GS activation. Treatment of glycogen-depleted myoblasts with either the MEK inhibitor, PD098059, or the PI 3-kinase inhibitor wortmannin partly reduced the activity state of GS obtained in response to glucose readministration. However, this is essentially attributable to these inhibitors reducing the basal activity state of GS, as previously observed (13). Therefore, these findings indicate that the mechanisms involved in the activation of GS by glucose are independent of the activity of PI 3-kinase, the classical mitogen-activated protein kinase pathway, and the rapamycin-sensitive pathway leading to activation of p70^{s6k}. Furthermore, during glucose readministration, there was no observable decrease in the activity of GSK-3 and no detectable phosphorylation of that protein, as detected by phosphospecific anti–GSK-3 antibodies (Fig. 6). No changes in the activity of protein kinase B were detected (not shown). Thus, the mechanism underlying the effects of glucose/glycogen are apparently distinct from those utilized by insulin.

Glucose-6-phosphate (G6P) is a potent allosteric activator of GS (19), whereas a number of small metabolites, such as ATP, ADP, and AMP, are capable of inhibiting GS activity (20). To confirm that the observed alterations in the fractional activity of GS are because of covalent modification and not carryover of allosteric activators or inhibitors, cell extracts were fractionated using Bio-Spin P-6 polyacrylamide gel spin columns to remove molecules with a mass <6 kDa. Use of ^{14}C-glucose-1-phosphate as a marker indicated that >95% of small metabolites were removed by this process. After refeeding of glycogen-depleted cells, fractionation of extracts failed to significantly alter GS activity ratio (0.255 ± 0.011 before fractionation and 0.283 ± 0.003 after fractionation), indicating that activation of GS was not because of allosteric activation by G6P or effects of other small molecules.

The activity of endogenous PPs against GS was then measured in extracts from control and glycogen-depleted cells. Because it has been demonstrated that GS becomes a better substrate for PPs in the presence of G6P (21), this was carried out over a range of G6P concentrations. No differences were found in the rate of activation of GS by endogenous phosphatases in extracts prepared from control and glycogen-depleted cells. For example, at a physiological concentration of 0.25 mmol/l G6P in the extract, the effect of endogenous phosphatases on GS activity in extracts of cells preincubated in the presence of glucose led to an increase of 3.44 ± 0.29 pmol · min^{-1} · mg $^{-1}$ GS activity in 30 min, compared with an increase of 3.85 ± 0.60 pmol · min^{-1} · mg $^{-1}$ in 30 min in cells preincubated in the absence of glucose for 5 h. Therefore, glycogen depletion does not appear to stimulate phosphatase activity against GS, at least when measured subsequently in cell extracts.

DISCUSSION

The results presented here demonstrate dramatic effects on both glycogen synthesis and glycogen synthase after glucose re-administration to human myoblasts that have previously been depleted of glycogen.

The activation of GS is observed by the use of an assay optimized to detect activity changes resulting from phosphorylation/dephosphorylation of the enzyme in hepatocytes (17). This is expressed as a fractional activity, i.e., activity in the presence of 0.1 mmol/l G6P as a fraction of the activity in 10 mmol/l G6P. Similar methodologies have been applied to studies of GS activity in muscle, where activity has been reported as a fractional velocity or as A0.5, the G6P concentration required for half maximal activity (22).

The molecular mechanism responsible for this stimulatory effect of glucose is a primary concern. The alterations in the fractional activity of GS are almost certainly because of a change in the phosphorylation state of the enzyme, as the removal of small activatory/inhibitory molecules from cell extracts failed to alter the elevated GS activity. Inhibition of GSK-3 is thought to be the key event in the activation of muscle GS by insulin; however, selective inhibitors of the known insulin-stimulated pathways leading potentially to GSK-3 inhibition failed to prevent the activation of GS during the refeeding of glucose to glycogen-depleted cells (Fig. 5). Furthermore, no inhibition or phosphorylation of GSK-3 was observed in response to glucose (Fig. 6). However, the involvement of GSK-3 inhibition in this process cannot be completely ruled out, as GSK-3 could be inhibited by an alternative, yet unidentified, mechanism not involving phosphorylation of the single serine residue recognized by the phosphospecific antibodies and not being retained when the enzyme is assayed in cellular extracts. In that regard, allosteric regulation of GSK-3 by glycogen or small metabolites must be considered. A recent study performed in rat skeletal

muscle demonstrated an exercise-induced increase in GS activity accompanied by inactivation of GSK-3 (23). However, in this model, GSK-3 inactivation was not associated with phosphorylation of the serine 9/21 (α/β) residues, and it was suggested that glycogen content might regulate GSK-3 activity through direct allosteric activation of the kinase by the glycogen particle, although no evidence was offered to support this hypothesis.

The activity of GS is dependent on the relative contribution of GSK-3 and PP1 in determining the phosphorylation state of the enzyme. Phosphorylation of the glycogen-binding subunit of PP1 has been postulated as a mechanism for activation of that enzyme in response to insulin; however, a growing body of evidence suggests that this is not the case (rev. in [1]). After exercise in human muscle, no increase in total or glycogen-associated PP activity was observed; however, the rate of reactivation of GS by endogenous phosphatases was increased, suggesting that GS became a better substrate for PP1 after exercise (24). In the present work, no increase in the phosphatase activity directed toward GS was observed after the glycogen depletion of human myoblasts. However, it is possible that PPs, principally PP1, can alter GS activity in vivo, independent of changes in the intrinsic activity of the phosphatase detected in in vitro assays. For example, effects of changes in intracellular G6P concentration to enhance the ability of PP1 to dephosphorylate GS (21), or the effect of overexpression of a glycogen-binding subunit of PP1 in CHO cells, which results in an increase in basal and insulin-stimulated glycogen synthesis (25), would not be detected as a change in phosphatase activity in cell extracts.

Work in rat adipocytes has previously shown that glucose can activate GS in the absence of insulin (26), an effect attributed to increased glucose transport leading to accumulation of G6P, which can then increase PP activity toward GS. The extent to which such a mechanism contributes to the observations in muscle reported in this study remains to be established, although studies in mouse diaphragm have demonstrated a modest stimulatory effect of glucose on GS activity (27). The model from rat adipocytes does not account for the effect of glycogen stores on the activation, and a recent study in human myotubes has indicated that glycogen repletion, after glucose administration to cells that have been depleted of glycogen by overexpression of glycogen phosphorylase, is not dependent on increases in intracellular G6P concentration (28).

However, metabolic flux through G6P has been suggested as the hub of coordinate regulation of muscle glycogen synthesis (29); changes in GS activity are a consequence of alterations in intracellular G6P concentration, a result of increased glucose uptake and hexokinase activity. G6P is an allosteric activator of GS, but also serves to make GS a better substrate for dephosphorylation by PPs (21). Low glycogen content in vivo could increase glucose uptake and hexokinase activity, resulting in an increase in intracellular G6P concentration and consequently in an increase in GS activity, although no obvious mechanism for this is known.

A further consideration is whether glycogen depletion followed by glucose refeeding alters the distribution of GS and its regulatory proteins within the myoblast. PP1, GSK-3, and GS are all capable of redistributing within the cell in response to metabolic and hormonal stimuli (5,30,31). If glucose (or one of its metabolites) caused either increased interaction of GS and PP1 or decreased interaction of GS and GSK-3 in glycogen-depleted cells, this could explain the activation of GS in response to this nutrient.

An exercise-induced increase in glycogen resynthesis after glycogen depletion can only occur if adequate substrate is available. Indeed, after glucose deprivation of cultured human muscle cells, there was a twofold increase in the subsequent rate of 2-deoxyglucose uptake. Increased glucose transporter numbers at the plasma membrane has been suggested as the mechanism for increased glucose uptake postexercise, and in rat skeletal muscle, a 1.8-fold increase in glucose transporters found at the plasma membrane was observed after exercise (32). The relationship between glycogen and membrane permeability to glucose is apparently reciprocal, with increasing glycogen content postexercise leading to a decrease in glucose uptake (33).

In summary, the work reported here has highlighted the important role of glucose in stimulating glycogen synthesis in muscle after glycogen depletion. Recent elegant work using ^{31}P nuclear magnetic resonance to study glycogen metabolism in human muscle in vivo is yielding important information on the effects of glycogen on glycogen synthesis (34). However, work with human muscle cells in culture has the benefit of providing a means of independently varying glycogen content, extracellular glucose concentration, and insulin levels. The data reported here are consistent with work in human muscle in vivo showing activation of GS after exercise-mediated glycogen depletion (22), leading to glycogen repletion in an insulin-independent phase (10). The molecular mechanism leading to the activation of GS by glucose after glycogen depletion is as yet undefined, but is apparently distinct from the upstream signaling mechanisms used in the actions of insulin on this parameter. Recent evidence suggests that AMP-activated protein kinase may play a role in insulin-independent exercise-stimulated glucose uptake (35). The possibility that it is also involved in the stimulation of glycogen synthesis after glycogen depletion is an attractive one, although no evidence is currently available to support this. Clearly, how the two different signaling pathways interact has implications for the overall control of glycogen synthesis in control subjects and patients with type 2 diabetes.

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