Muscle glycogen inharmoniously regulates glycogen synthase activity, glucose uptake, and proximal insulin signaling

Jørgen Jensen, Einar Jebens, Erlend O. Brennesvik, Jérôme Ruzzin, Maria A. Soos, Ellen M. L. Engebretsen, Stephen O’Rahilly, and Jonathan P. Whitehead. Muscle glycogen inharmoniously regulates glycogen synthase activity, glucose uptake, and proximal insulin signaling. Am J Physiol Endocrinol Metab 290: E154–E162, 2006. First published August 23, 2005; doi:10.1152/ajpendo.00330.2005. —Insulin stimulates glucose uptake and incorporation of glucose into skeletal muscle glycogen contribute to physiological regulation of blood glucose concentration. In the present study, glucose handling and insulin signaling in isolated rat muscles with low glycogen (LG, 24-h fasting) and high glycogen (HG, refed for 24 h) content were compared with muscles with normal glycogen (NG, rats kept on their normal diet). In LG, basal and insulin-stimulated glycogen synthesis and glycogen synthase activation were higher and glycogen synthase phosphorylation (Ser645, Ser649, Ser653, Ser657) lower than in NG. GLUT4 expression, insulin-stimulated glucose uptake, and PKB phosphorylation were higher in LG than in NG, whereas insulin receptor tyrosyl phosphorylation, insulin receptor substrate-1-associated phosphatidylinositol 3-kinase 3-kine activity, and GSK-3 phosphorylation were unchanged. Muscles with HG showed lower insulin-stimulated glycogen synthesis and glycogen synthase activation than NG despite similar dephosphorylation. Insulin signaling, glucose uptake, and GLUT4 expression were similar in HG and NG. This discordant regulation of glucose uptake and glycogen synthesis in HG resulted in higher insulin-stimulated glucose 6-phosphate concentration, higher glycolytic flux, and intracellular accumulation of nonphosphorylated 2-deoxyglucose. In conclusion, elevated glycogen synthase activation, glucose uptake, and GLUT4 expression enhance glycogen resynthesis in muscles with low glycogen. High glycogen concentration per se does not impair proximal insulin signaling or glucose uptake. "Insulin resistance" is observed at the level of glycogen synthase, and the reduced glycogen synthase leads to increased levels of glucose 6-phosphate, glycolytic flux, and accumulation of nonphosphorylated 2-deoxyglucose.

glucose transporter 4; protein kinase B; glycogen synthase kinase-3; phosphorylation; glucose metabolism; glycolytic flux; rat

SKELETAL MUSCLE IS PARTICULARLY IMPORTANT in maintaining blood glucose homeostasis, as 70–90% of insulin-stimulated glucose uptake occurs in skeletal muscle, where it is incorporated into and stored as glycogen (37). The glycogen concentration in skeletal muscles is limited, and a feedback mechanism exists. It has been 40 years since the classic study of Danforth (8), which showed that increased glycogen concentration reduced glycogen synthase activation in both the presence and absence of insulin. More recently, studies have shown that glycogen concentration also influences glucose uptake in skeletal muscles (2, 9, 17, 18).

Among the most important functions of insulin are stimulation of glucose uptake and activation of glycogen synthesis (35). Insulin stimulates glucose uptake in insulin responsive tissues by promoting translocation of the facilitative glucose transporter GLUT4 from intracellular vesicles to the cell membrane (38) and activates glycogen synthase by dephosphorylation (6, 25). The initial steps in the signaling pathways for insulin-stimulated translocation of GLUT4 and activation of glycogen synthase are common. Upon insulin binding, the insulin receptor undergoes autophosphorylation and tyrosine phosphorlylates several proteins, most notably the insulin receptor substrates (IRS). These serve to recruit and, in many cases, activate SH2 domain-containing proteins (34). One such protein is the class I-A phosphatidylinositol (PI) 3-kinase (34). Activation of PI 3-kinase is essential for the propagation of many of insulin’s subsequent downstream signaling events and metabolic effects (36). Downstream of PI 3-kinase lies the Ser/Thr protein kinase B (PKB, otherwise called Akt), which is activated by phosphorylation of at least two sites (1). PKB activation appears to be essential for efficient insulin-stimulated glucose uptake (21), although the molecular link between PKB and glucose uptake remains to be defined. One candidate is the recently identified GTPase-activating protein AS160 (32). By contrast, the link between PKB and glycogen synthesis is established. PKB phosphorylates and inhibits GSK-3 directly, which contributes to increased activation of glycogen synthase (7).

Regulation of glycogen synthase activity is complex, involving phosphorylation of nine amino acids by different kinases and dephosphorylation by protein phosphatase-1 (PP-1) (6, 25). Although the inverse relationship between glycogen concentration and glycogen synthase activity is well established (8, 27), mechanisms for the inhibition of glycogen synthase activity are poorly defined. Inability of insulin to activate glycogen synthase precedes the development of type 2 diabetes (33), leading to the suggestion that dysregulation of glycogen synthase may promote type 2 diabetes.

We (18) previously reported that reducing glycogen content by fasting doubled insulin-stimulated glucose uptake in isolated rat skeletal muscles, whereas increasing glycogen content by refeeding did not significantly influence maximal insulin-stimulated glucose uptake. In light of the strong reduction of...
glycogen synthase activation in muscles with high glycogen content (8), we hypothesized that these apparently discordant effects of glycogen on glucose uptake and glycogen synthase would alter glucose handling. Hence, the first aim of the present study was to investigate alterations in glucose metabolism in muscles with different glycogen concentrations. Furthermore, because the mechanisms for regulation of glycogen synthase activation by glycogen are poorly understood, the second aim of the present study was to investigate regulation of PKB, GSK-3, and glycogen synthase phosphorylation in muscles with different glycogen concentrations.

RESEARCH DESIGN AND METHODS

Materials. Reagents were from Sigma (St. Louis, MO and Poole, Dorset, UK) unless otherwise stated. IRS-1 antibody has been described previously (23). The anti-phosphotyrosine (4G10), anti-GSK-3α and β, and anti-phospho-GSK-3α (Ser21) antibodies were from Upstate Biotechnology (Lake Placid, NY). The anti-PKB and anti-phospho-PKB (Ser473) antibodies were from Upstate Biotechnology and New England Biolabs (Hertfordshire, UK), respectively. In our hands, these antibodies recognize all three PKB isoforms with similar efficiency (determined using recombinant hemagglutinin-tagged PKBα, PKBβ, and PKBγ). Anti-phospho-GSK-3α/β (Ser21) was from Cell Signaling (Beverly, MA). Anti-glycogen synthase was a gift from Oluf Pedersen (Copenhagen, Denmark). Anti-GLUT4 and anti-GLUT1 were a gift from David E. James (Sydney, Australia). Anti-GLUT4 and anti-GLUT1 were a gift from David E. James (Sydney, Australia). Anti-phospho-glycogen synthase (Ser645, Ser649, Ser653, Ser657) was from Oncogene (Darmstadt, Germany).

Animals. Male Wistar rats (Bk1-Wist) were obtained from B & K Universal (Nittedal, Norway) and housed in our laboratory animal facilities for ≥1 wk before the experiment. Room temperature was 21°C, humidity was ≥55%, and a 12:12 light-dark cycle (light from 6 AM to 6 PM) was set. The rats had free access to standard rat chow (B & K Universal, Grimston, UK) and tap water. The experiments were performed during the light cycle (between 10 AM and 2 PM), and the weights of the rats on the day of the experiment were 120-150 g. Experiments and procedures were approved by official authorities and performed in accordance with the laws and regulations controlling experiments on live animals in Norway and the European Convention for the Protection of Vertebrate Animals used in Experimental and Other Scientific Purposes.

The glycogen concentration was manipulated to achieve muscles with low (LG), normal (NG), and high glycogen (HG) concentrations as described previously (18). In brief, rats with NG were maintained on their normal diet before experiments (control). Rats with HG were fasted for 24 h and then allowed access to a normal diet for 24 h before experiments (fasted/refed).

Muscle preparation and incubation. The rats were anesthetized with an injection of ≈10 mg of pentobarbital sodium (50 mg/ml) intraperitoneally, and the epimisolear muscles were dissected out and suspended on holders at their approximate resting length. The muscles were then placed in test tubes, and gas (95% O2-5% CO2) was continuously bubbled through the incubation buffer, as described previously (3). The muscles were preincubated for 30–50 min in 3.5 ml of modified Krebs-Henseleit buffer containing (in mM) 116 NaCl, 4.6 KCl, 1.16 KH2PO4, 25.4 NaHCO3, 2.5 CaCl2, 1.16 MgSO4, 5.5 glucose, 2 sodium pyruvate, and 5 HEPES [N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)] and 0.1% bovine serum albumin (fraction V, Sigma), pH 7.4. All incubations were performed at 30°C without or with the addition of a maximal insulin dose of 10 μU/ml (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) for 30 min.

Glucose uptake. Glucose uptake was measured as described previously (3). In brief, 0.25 μCi/ml 2-deoxy-β-[1-14C]glucose (25.5 Ci/mmol; DuPont-NEN) were added to the buffer (containing 5.5 mM glucose), and glucose uptake was calculated from the intracellular accumulation of 2-deoxy-β-[1-14C]glucose. After incubations, the muscles were removed from the holders, blotted on filter paper, and frozen in liquid nitrogen. For analysis of glucose uptake, the muscles were freeze-dried, weighed, and dissolved in 600 μl of 1 M KOH for 20 min at 70°C. Of the digest, 400 μl were added to 3 ml of scintillation solution (Hionic-Fluor, Packard), mixed, and counted for radioactivity (Tri-Carb 460C; Packard Instruments, Downers Grove, IL).

Accumulation of nonphosphorylated glucose. Accumulation of nonphosphorylated 2-deoxyglucose was determined as described previously (3). In brief, muscles were incubated with 0.25 μCi/ml 2-deoxy-β-[1,2-3H(N)]glucose (25.5 Ci/mmol) and 0.1 μCi/ml β-[1-14C]mannitol (51.5 Ci/mmol; DuPont-NEN) containing 5.5 mM glucose for 30 min. After homogenization in perchloric acid, 2-deoxy-β-[1-3H]glucose 6-phosphate was separated on a Dowex column, and free glucose was calculated.

Glycolytic flux. Glycolytic flux was determined from release of 3H2O from β-[5-3H(N)]glucose during glycolysis (16). Muscles were incubated for 60 min at 30°C in small vials, as described previously (18), in 1.1 ml of buffer containing 1.0 μCi/ml β-[5-3H(N)]glucose (20.0 Ci/mmol, DuPont-NEN) with and without 10 μU/ml insulin. After incubation, 3H2O was separated from β-[5-3H]glucose by water equilibration in sealed vials at 37°C for 92 h as described (4).

Glycogen synthesis. Glycogen synthesis was determined from incorporation of [14C]glucose into glycogen during 60 min of incubation in the presence of 5.5 mM glucose and 0.2 μCi/ml [14C]glucose (20.0 Ci/mmol, DuPont-NEN) as described previously (13).

Glycogen concentration. For analysis of glycogen, 100 μl of the KOH digest were neutralized with 25 μl of 7 M acetic acid, and 500 μl of 0.3 M acetate buffer (pH 4.8) containing 0.2 U/ml amyloglucosidase (Boehringer Mannheim) were added. The glycogen was hydrolyzed at 37°C for 3 h, and glucose units were analyzed as described previously (20).

Glycogen synthase activity. Glycogen synthase activity was measured with the filter paper method that we have described previously (20). The final concentration of uridine diphosphate glucose was 30 μM with 0.5 μCi/ml [14C]uridine diphosphate glucose (287.4 Ci/mmol, DuPont-NEN) added. Glycogen synthase total activity was determined at the saturation concentration of glucose 6-phosphate (8 mM). Glycogen synthase activity was also measured at 0.17 mM glucose 6-phosphate, and fractional activity was calculated as the activity at 0.17 mM glucose 6-phosphate in percent total activity.

Western blot analyses. Immunoblotting was performed as described previously (42). In brief, total muscle protein lysates (∼50 μg) were resolved by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA), and membranes were probed with the appropriate primary and secondary antibodies. Antibody binding was detected by enhanced chemiluminescence (Amer sham Pharmacia Biotech, Buckinghamshire, UK) or phosphorimaging using a Fujix Bas 2000 PhosphorImager (Fuji Photo Film, Tokyo, Japan).

Immunoprecipitation and assay of PI 3-kinase activity. Immunoprecipitations were performed on ∼350 μg of muscle protein for 2 h, and PI 3-kinase assays were carried out essentially as described previously (42).

Statistical analysis. The data are presented as means ± SE. One-way ANOVA was performed to investigate differences between experimental groups. Least significant difference tests were performed post hoc. P < 0.05 was considered as significant.

RESULTS

Changes in glycogen metabolism associated with altered glycogen stores. Fasting for 24 h decreased glycogen content in epimisolear muscles by ∼50% (Table 1, LG). When 24-h-
fasted rats were allowed access to normal chow for another 24 h, glycogen content increased to double that observed in rats maintained on a normal diet (Table 1, HG and NG). As the glycogen concentration increased, the rate of glycogen synthesis decreased both in the absence and in the presence of insulin (Fig. 1). The potency of the effect of glycogen concentration on glycogen synthesis is highlighted by the comparable rate of glycogen synthesis in muscles with LG incubated without insulin and in muscles with HG treated with insulin. Whereas insulin increased the rate of glycogen synthesis in all groups about fivefold, the absolute increase in response to insulin varied depending on the glycogen concentration, and the rate of insulin-stimulated glycogen synthesis was reduced by 70% in muscles with HG compared with those with NG.

In accordance with the inverse correlation between glycogen concentration and glycogen synthase, glycogen synthase fractional activity decreased as the glycogen concentration increased in both the absence and presence of insulin (Fig. 2). Insulin increased glycogen synthase fractional activity in muscles with LG and NG, but no significant increase was observed in muscles with HG. Furthermore, glycogen synthase fractional activity was higher in muscles with LG in the absence of insulin compared with that in muscles with NG or HG with and without insulin (P < 0.05).

Typically, phosphorylations of glycogen synthase at sites phosphorylated by GSK-3 (Ser\(^{645}\), Ser\(^{649}\), Ser\(^{653}\), Ser\(^{657}\), named site-3 phosphorylation in the present paper) are prominent inhibitors of glycogen synthase activity (30, 39). Consistent with this, site-3 phosphorylation was decreased in muscles with LG compared with those with NG (Fig. 3). Insulin promoted a significant decrease in site-3 phosphorylation in all groups, including those with HG. Surprisingly, glycogen synthase phosphorylation was lower in muscles with HG than in those with NG in the absence of insulin despite lower glycogen synthase activity. Furthermore, insulin decreased glycogen synthase phosphorylation in muscles with HG to a level similar to that observed in muscles with NG, but insulin was unable to activate glycogen synthase significantly (Figs. 2 and 3).

The electrophoretic mobility of glycogen synthase, as determined by immunoblotting using an antibody against glycogen synthase, differed between groups and within groups depending on treatment (Fig. 3). In control muscles with HG, glycogen synthase migrated as a single discrete band when insulin was absent. After insulin treatment, the intensity of this band was reduced concomitantly with the appearance of an additional lower band. This band shift probably reflects dephosphorylation of a fraction of glycogen synthase. In muscles with LG, the band shift was clearly visible in muscles even without insulin treatment. There was no detectable band shift in muscles with HG either before or after insulin treatment, with only the upper band visible.

The expression levels of glycogen synthase did not vary significantly between groups (Table 1). Total glycogen synthase activity, which was not influenced by insulin, was similar in muscles with LG and NG, but slightly reduced in muscles with HG (Table 1).

![Fig. 1. Alterations in glycogen synthesis in muscles with different glycogen concentrations. Mean rate of glycogen synthesis in epitrochlearis muscles with low (LG), normal (NG), and high glycogen (HG) incubated without (open bars) or with (filled bars) 10 mU/ml of insulin for 60 min; n = 6–9 for each group. *Significant difference between muscles incubated with and without insulin; †significantly higher than muscles with NG and HG incubated without insulin; ‡significantly different from muscles with LG and HG.

![Fig. 2. Changes in glycogen synthase activity in muscles with different glycogen concentrations. Mean glycogen synthase fractional activity in epitrochlearis muscles with LG, NG, and HG after 30-min incubation without (open bars) or with (filled bars) 10 mU/ml of insulin; n = 6–9 for each group. *Significant difference between muscles incubated with and without insulin; †significantly higher than muscles with NG and HG incubated without insulin; ‡significantly different from muscles with LG and HG.

Table 1. Glycogen concentration, glycogen synthase activity, and protein expression in epitrochlearis muscles with LG, NG, and HG

<table>
<thead>
<tr>
<th>Glycogen, mmol/kg dw</th>
<th>LG</th>
<th>NG</th>
<th>HG</th>
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<tr>
<td>5.7 (12)ab</td>
<td>180.6 ± 8.5 (15)</td>
<td>361.3 ± 39.4 (12)</td>
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<tr>
<td>GS activity, mmol/kg dw/min</td>
<td>2.5 ± 0.2 (12)*</td>
<td>2.5 ± 0.1 (18)*</td>
<td>2.0 ± 0.1 (12)</td>
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<tr>
<td>Protein expression</td>
<td>GS</td>
<td>PKB</td>
<td>GSK-3α</td>
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<tr>
<td>95.2 ± 5.9 (9)</td>
<td>118.9 ± 7.2 (10)*</td>
<td>110.6 ± 8.9 (8)</td>
<td>103.0 ± 7.5 (8)</td>
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<td>100.0 ± 5.7 (9)</td>
<td>100.0 ± 3.1 (10)</td>
<td>100.0 ± 9.4 (8)</td>
<td>100.0 ± 2.4 (8)</td>
</tr>
<tr>
<td>104.3 ± 6.1 (9)</td>
<td>92.6 ± 4.7 (10)</td>
<td>97.8 ± 6.0 (8)</td>
<td>93.5 ± 3.1 (8)</td>
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</table>

Values are means ± SE; n = 12 for each group. aSignificant difference between muscles incubated with and without insulin; †significantly higher than muscles with HG incubated without insulin; ‡significantly different from muscles with LG and HG.
reduced glycogen synthesis observed in muscles with HG (Fig. 1). GLUT1 expression did not show any significant changes in muscles with different glycogen concentrations (Fig. 4B). By contrast, expression of GLUT4 was increased by 90% in muscles with LG compared with muscles with NG (Fig. 4B). Twenty-four-hour refeeding reduced GLUT4 content such that GLUT4 protein in muscles with HG was similar to that observed in muscles with NG (Fig. 4B). Therefore, insulin-stimulated glucose uptake mirrored GLUT4 expression.

To investigate the fate of the glucose, we measured the levels of glucose 6-phosphate and glycolytic flux. Glucose 6-phosphate levels were similar in all groups in the absence of insulin (Table 2). Insulin increased glucose 6-phosphate concentration in muscles with HG to almost double the concentrations in other groups (Table 2). Twenty-four-hour refeeding reduced GLUT4 content such that GLUT4 protein in muscles with HG was similar to that observed in muscles with NG (Fig. 4B). Therefore, insulin-stimulated glucose uptake mirrored GLUT4 expression.

Investigation of insulin signaling in muscles with altered glycogen and glucose metabolism. Insulin signaling was investigated in an attempt to understand how the changed glucose handling occurred. Insulin-stimulated autophosphorylation of the insulin receptor represents the first step in the insulin-signaling cascade and was comparable in all groups (Fig. 5A). A key event in the proximal insulin-signaling cascade, particularly in skeletal muscle, is the recruitment and activation of PI 3-kinase by tyrosyl-phosphorylated IRS-1. Insulin-stimulated IRS-1-associated PI 3-kinase activity was increased fivefold in all groups (Fig. 5B), suggesting that insulin-stimulated tyrosine phosphorylation and recruitment of PI 3-kinase to IRS-1 was not affected by changes in glycogen concentration. One of the downstream effectors of PI 3-kinase that is involved in both glycogen synthesis and glucose uptake is the Ser/Thr kinase PKB. Although insulin stimulated a marked increase in PKB phosphorylation (Ser473) in all groups, this was more than twice as high in muscles with LG compared with muscles with NG or HG (Fig. 6). Insulin also promoted a greater PKB band shift in muscles with LG compared with muscles with NG or HG (data not shown), providing further support that a greater proportion of PKB was phosphorylated in response to insulin treatment in muscles with LG. Whereas expression of PKB was moderately increased (~20%, P < 0.05) in muscles with LG (Fig. 6 and Table 1), this increase is unlikely to account for the increased insulin-stimulated phosphorylation. PKB expres-

Fig. 3. Alterations in glycogen synthase phosphorylation in muscles with different glycogen concentrations. A, top: representative blots showing glycogen synthase (GS) site-3 phosphorylation. Bottom: GS mobility. B: quantitation of site-3 phosphorylation; n = 12 for each group. *Significant difference between muscles incubated with and without insulin; †significantly higher than muscles with NG and HG incubated without insulin; ‡significantly different from LG and NG incubated without insulin.

Fig. 4. Glucose uptake and expression of glucose transporters in muscles with different glycogen concentrations. A: basal (open bars) and insulin-stimulated glucose uptake (filled bars) in muscles with LG, NG, and HG; n = 6 for each group. B: expression of GLUT1 (hatched bars) and GLUT4 (cross-hatched bars) in muscles with LG, NG, and HG; n = 12 for each group. *Significant difference from muscles incubated without insulin; †significantly different from NG and HG.
tion and phosphorylation were similar in muscles with NG and HG. These data suggest that insulin-stimulated phosphorylation of PKB correlates with increased insulin-stimulated glucose uptake in muscles with LG compared with muscles with NG or HG.

Next, we examined the expression and phosphorylation of GSK-3, a direct substrate of PKB and upstream kinase of glycogen synthase. Expression of GSK-3α or GSK-3β was unchanged in the treatment groups (Fig. 7 and Table 1). GSK-3α Ser21 phosphorylation (which was detected by two commercially available antibodies) was similar in all groups in the absence of insulin (Fig. 7, A and B). Insulin-stimulated GSK-3α Ser21 phosphorylation was significantly higher in muscles with LG than in muscles with HG. GSK-3β Ser9 phosphorylation (which was detected only by the antibody from Cell Signaling) appeared to be constitutively phosphorylated (Fig. 7, A and C). Insulin promoted a twofold increase in GSK-3β Ser9 phosphorylation in all groups, and, like GSK-3α, insulin-stimulated GSK-3β Ser9 phosphorylation was significantly higher in muscles with LG compared with HG.

**DISCUSSION**

In the present study, we have investigated how alterations in skeletal muscle glycogen concentration are associated with glucose handling and insulin signaling. In muscles with HG, proximal insulin signaling, glucose uptake, and the extent of insulin-stimulated glycogen synthase site-3 dephosphorylation by 10.220.33.3 on August 4, 2017 http://ajpendo.physiology.org/ Downloaded from http://ajpendo.physiology.org/ by 10.220.33.3 on August 4, 2017

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**Table 2. Glucose 6-phosphate, glycolytic flux, and free 2-deoxyglucose in muscles with LG, NG, and HG**

<table>
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<tr>
<th>Glucose 6-phosphate</th>
<th>Basal</th>
<th>Insulin</th>
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<tbody>
<tr>
<td>LG</td>
<td>0.26±0.03 (8)</td>
<td>0.40±0.08 (8)</td>
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<td>NG</td>
<td>0.36±0.05 (8)</td>
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<tr>
<td>HG</td>
<td>0.44±0.07 (8)</td>
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<th>Non-phospho-2-deoxyglucose</th>
<th>Basal</th>
<th>Insulin</th>
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</thead>
<tbody>
<tr>
<td>LG</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NG</td>
<td>0.85±0.18 (11)</td>
<td>1.71±0.21 (11)†</td>
</tr>
<tr>
<td>HG</td>
<td>0.84±0.05 (12)</td>
<td>2.33±0.20 (12)*,†</td>
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<tr>
<th>Glycolytic flux</th>
<th>Basal</th>
<th>Insulin</th>
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<tbody>
<tr>
<td>LG</td>
<td>0.99±0.12 (8)</td>
<td>1.79±0.14 (8)*</td>
</tr>
<tr>
<td>NG</td>
<td>0.96±0.06 (15)</td>
<td>1.76±0.11 (15)*</td>
</tr>
<tr>
<td>HG</td>
<td>0.84±0.05 (12)</td>
<td>2.33±0.20 (12)*,†</td>
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Values are means ± SE in mmol/kg dry wt; no. of muscles given in parentheses. ND, not determined. *Significantly higher than basal; †significantly higher than HG.

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**Fig. 5. Proximal insulin signaling in muscles with different glycogen concentration.**

A: tyrosine phosphorylation of the insulin receptor (PY-IR) in muscles with LG, NG, and HG incubated without (open bars) or with (filled bars) 10 mU/ml insulin. Top: representative immunoblot showing tyrosine phosphorylation of the insulin receptor β-subunit. Bottom: quantitation of insulin receptor tyrosine phosphorylation; n = 7–9 for each group. B: Insulin receptor substrate (IRS)-1-associated PI 3-kinase activity in muscles with LG, NG, and HG incubated without (open bars) or with (filled bars) 10 mU/ml insulin; n = 9–10 for each group.

**Fig. 6. Altered PKB phosphorylation in muscles with different glycogen concentrations.**

A: top: representative blot of PKB Ser473 phosphorylation in muscles with LG, NG, and HG incubated without or with 10 mU/ml insulin. Bottom: expression of PKB in the 3 groups. B: quantitation of PKB Ser473 phosphorylation in muscles with LG, NG, and HG incubated without (open bars) or with (filled bars) 10 mU/ml insulin; n = 11–14 for each group. *Significantly higher than NG and HG.
were all comparable with muscles with NG. By contrast, insulin-stimulated glycogen synthesis, glycogen synthase activation, and glycogen synthase band shift were all reduced, suggesting inhibition of glycogen synthase by mechanisms independent of site-3 phosphorylation. In the face of unaltered glucose uptake in muscle with HG, both glucose 6-phosphate and nonphosphorylated glucose accumulated, and glycolytic flux was increased. Compared with NG, muscle with LG showed increased glycogen synthesis, glycogen synthase activation, glycogen synthase dephosphorylation, glucose uptake, GLUT4 expression, and insulin-stimulated PKB phosphorylation.

The activation of glycogen synthase in muscles with LG was increased compared with that observed in muscles with NG. In fact, glycogen synthase fractional activity was higher in muscles with LG in the absence of insulin than in muscles with NG in the presence of insulin, highlighting that glycogen is a stronger regulator than insulin. Our observation that the rate of glycogen synthesis and the activation of glycogen synthase correlate inversely with the glycogen concentration in both the absence and presence of insulin, is consistent with other studies (8, 19, 27, 29). Glycogen synthase activity is regulated by phosphorylation/dephosphorylation of at least nine sites, each of which may contribute to the regulation of glycogen synthase activity to varying degrees, but the sites phosphorylated by GSK-3 are prominent regulators (6, 25). We observed lower site-3 phosphorylation in muscles with LG than in muscles with NG in the absence of insulin, and site-3 phosphorylation was further reduced by insulin. The commercial site-3 antibody used in the present study was raised against a phosphopeptide sequence containing four phosphorylated serine residues (Ser645, Ser649, Ser653, Ser657, also named sites 3a, 3b, 3c, and 4, respectively). A thorough understanding of the precise characteristics of this antibody-antigen interaction is lacking, limiting the interpretation of our observations. However, in muscles with NG, glycogen synthase exhibits an insulin-induced band shift, consistent with dephosphorylation, and the reduced site-3 phosphorylation was paralleled with increased fractional activity. In muscles with LG, reduced site-3 phosphorylation also occurred simultaneously with increased band shift and glycogen synthase activation, which is consistent with the notion that site-3 dephosphorylation increases glycogen synthase fractional activity (6, 25).

Surprisingly, glycogen synthase site-3 phosphorylation was either lower (in the absence of insulin) or comparable (after insulin treatment) in muscles with HG compared with that observed in muscles with NG. This contrasts with the lower glycogen synthase fractional activity in muscles with HG compared with NG. Therefore, phosphorylation of glycogen synthase at the sites phosphorylated by GSK-3 does not correlate with reduced glycogen synthase activity in HG. Furthermore, glycogen synthase band shift did not occur during insulin stimulation in muscles with HG despite decreased site-3 phosphorylation. These novel findings indicate that glycogen synthase may be phosphorylated on other sites or subjected to additional forms of posttranslational modification in muscles with HG. Recently, Højlund et al. (15) reported normal insulin-stimulated glycogen synthase dephosphorylation, using an antibody raised against sites 3a and 3b, in muscles from subjects with type 2 diabetes without concomitant activation of glycogen synthase. Unexpectedly, insulin promoted increased phosphorylation at site 2a in these muscles (15). These data are consistent with our observations in muscles with HG, where insulin reduced site-3 phosphorylation but failed to activate glycogen synthase or promote a mobility shift.

Muscles with LG exhibited increased basal and insulin-stimulated glucose uptake. GLUT4 expression, but not GLUT1, was also increased in muscles with LG. Previous investigations have also reported fasting-induced increases in GLUT4 expression (5) and insulin-stimulated glucose uptake in skeletal muscles (18, 44). GLUT4 is expected to transport the majority of glucose into skeletal muscles, and we find it likely that the elevated glucose uptake observed in muscles with LG, at least
in part, is a consequence of increased GLUT4 expression. This is supported by Derave et al. (10), who reported increased cell surface GLUT4 content and glucose uptake in muscles with LG in the absence of insulin. However, no direct link between glycogen content and GLUT4 translocation has been demonstrated. Also of note is the previous observation that fasting reduces GLUT4 expression in adipose tissue (5), and such cell type-specific regulation of GLUT4 expression in response to fasting might serve to channel glucose into muscle glycogen when carbohydrate again becomes available.

The major problem when attempting to resolve whether increased glycogen concentration per se regulates glucose transport is that the manipulations performed to vary glycogen content may influence insulin sensitivity via other mechanisms. Insulin-stimulated glucose uptake was reduced in muscle either when glycogen content was increased by overexpression of GLUT1 or after exposure to high concentrations of insulin and glucose (5). By contrast, when glycogen content was increased, e.g., by overexpression of glycogen synthase or by fasting/refeeding, this did not obstruct basal or insulin-stimulated glucose uptake (9, 12, 18). The latter studies suggest that elevated glycogen content per se is unlikely to inhibit glucose uptake directly. Furthermore, our finding that GLUT4 expression was normalized after 24 h of refeeding agrees with previous reports (5) and appears to be consistent with the similar rate of insulin-stimulated glucose uptake in HG and NG.

The discordant regulation of glucose uptake and glycogen synthesis in muscles with HG, where glycogen synthesis becomes the rate-limiting step, was also reported by Laurent et al. (24). Further investigations revealed elevated glucose 6-phosphate in muscle with HG, compared with NG and LG, during insulin treatment. Glucose 6-phosphate allosterically activates glycogen synthase and inhibits hexokinase (25, 43). Hexokinase has a low Ki for glucose 6-phosphate (43), and the accumulation of nonphosphorylated 2-deoxyglucose supports that hexokinase may be inhibited in muscles with HG. Tracer amounts of 2-deoxyglucose are widely used to estimate the rates of glucose uptake in skeletal muscles, and a lumped constant close to 1 has been reported (11). In the present study, we observed a good correlation between the estimated glucose uptake rates and the combined rates for glycogen synthesis and glycolytic flux in muscles with LG and NG. By contrast, estimated glucose uptake was much greater than the sum of glycogen synthesis and glycolysis in muscles with HG. We are presently unable to provide an explanation for this discrepancy.

Despite the possible inhibition of hexokinase, insulin still increased glycogen synthesis and glycolytic flux in muscles with HG. Laurent et al. (24) previously reported that glycolytic flux is elevated in muscles with HG together with elevated glucose 6-phosphate. Phosphoglucose isomerase converts glucose 6-phosphate to fructose 6-phosphate in a near-equilibrium reaction (26); hence, fructose 6-phosphate will increase with subsequent activation of phosphofructokinase (40), the rate-limiting step in glycolysis. Elevated glucose 6-phosphate in muscles with HG may also facilitate insulin-stimulated glycogen synthesis, as glycogen synthesis occurs without significant activation of glycogen synthase activity in the present study (measured in vitro with fixed glucose 6-phosphate). Taken together, these observations provide novel mechanistic insights into how glucose handling is altered in muscles with HG.

Like glucose uptake, all aspects of insulin signaling were relatively unaltered in muscles with HG. Varying the glycogen concentration by fasting and fasting/refeeding did not influence proximal steps in insulin signaling, including insulin receptor phosphorylation and IRS-1-associated PI 3-kinase activation. PKB phosphorylation (Ser473) and GSK-3 phosphorylation were also normal in muscle with HG. On the other hand, in muscles with LG content, insulin-stimulated PKB phosphorylation (Ser473) was increased. These observations are consistent with previous reports of increased insulin-stimulated PKB activity and glucose uptake in muscle with LG (9, 22). The mechanism underlying the increased PKB phosphorylation and activation in muscle with LG is not immediately apparent. It appears unlikely that the 20% increase in total PKB expression explains the twofold increase in PKB phosphorylation. Because IRS-1-associated PI 3-kinase activity is normal in muscle with LG, it seems more likely that LG exerts its effects at a level between PI 3-kinase and PKB. It would be attractive to suggest that increased insulin-stimulated phosphorylation of PKB may contribute to the increased glucose uptake in muscles with LG. However, the antibodies used in the present study (PKB and phospho-PKB Ser473) recognize all isoforms of PKB, and we have not specifically shown increased phosphorylation of PKBβ, which seems to regulate insulin-stimulated glucose uptake (21). Future studies are required to characterize, in an isoform-specific manner, the mechanisms by which LG regulates expression and phosphorylation of PKB.

PKB phosphorylates GSK-3α and GSK-3β directly and reduces GSK-3 activity (7), but GSK-3α/β phosphorylation did not mirror PKB phosphorylation. Moreover, changes in glycogen concentration did not affect GSK-3 phosphorylation in the absence of insulin and had only minor effects in the presence of insulin. The present study, therefore, demonstrates that GSK-3 phosphorylation does not correlate with glycogen synthase activity or with glycogen synthase site-3 phosphorylation when glycogen content is varied. In muscles with LG, glycogen synthase was dephosphorylated in the absence of insulin without increased GSK-3 phosphorylation. Furthermore, in the presence of insulin, GSK-3 phosphorylation was not significantly elevated in muscles with LG compared with NG, despite further reduction (60%) in glycogen synthase site-3 phosphorylation. Collectively, these data suggest that regulation of glycogen synthase fractional activity in LG occurs via a mechanism that may be largely independent of changes in GSK-3, as is the case during exercise (31). This makes it appealing to suggest that activation of glycogen phosphatase PP-1 is increased in muscles with LG, reducing site-3 phosphorylation of glycogen synthase. Consistent with this, it has been reported that glycogen phosphatase activity correlates negatively with glycogen content (41). However, glycogen phosphatase activity is not altered in McArdle’s disease, where muscle glycogen is elevated (28), or in human muscle cells when glycogen content is decreased by glucose starvation (14).

Taken together, our data show that increased glycogen concentration per se does not impair proximal insulin signaling or glucose uptake but does result in changes in glucose metabolism. “Insulin resistance” is observed at the level of glycogen synthase despite normal total dephosphorylation of Ser645, Ser649, Ser653, and Ser657, and the reduced glycogen synthesis leads to increased levels of glucose 6-phosphate and
glycolytic flux. Over time, such alterations in glucose metabolism may promote intramuscular changes, resulting in impaired insulin signaling and glucose uptake. Hence, future work on glucose handling in rats with elevated muscle glycogen may provide important information about how nutrient oversupply is linked to impaired insulin action.

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