Additive effect of contraction and insulin on glucose uptake and glycogen synthase in muscle with different glycogen contents

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Lai YC, Zarrinpashneh E, Jensen J. Additive effect of contraction and insulin on glucose uptake and glycogen synthase in muscle with different glycogen contents. J Appl Physiol 108: 1106–1115, 2010. First published February 25, 2010; doi:10.1152/japplphysiol.00401.2009.—Insulin and contraction regulate glucose uptake and glycogen synthase (GS) via distinct mechanisms in skeletal muscles, and an additive effect has been reported. Glycogen content is known to influence both contraction- and insulin-stimulated glucose uptake and GS activity. Our study reports that contraction and insulin additively stimulate glucose uptake in rat epitrochlearis muscles with normal (NG) and high (HG) glycogen contents, but the additive effect was only partial. In muscles with low glycogen (LG) content no additive effect was seen, but glucose uptake was higher in LG than in NG and HG during contraction, insulin stimulation, and when the two stimuli were combined. In LG, contraction-stimulated AMP-activated protein kinase (AMPK) activity and insulin-stimulated PKB phosphorylation were higher than in NG and HG, but phosphorylation of Akt substrate of 160 kDa was not elevated correspondingly. GLUT4 content was 50% increased in LG (rats fasted 24 h), which may explain the increased glucose uptake. Contraction and insulin also additively increased GS fractional activity in NG and HG but not in LG. GS fractional activity correlated most strongly with GS Ser641 phosphorylation (R = 0.94, P < 0.001). GS fractional activity also correlated with GS Ser7,10 phosphorylation, but insulin did not reduce GS Ser7,10 phosphorylation. In conclusion, an additive effect of contraction and insulin on glucose uptake and GS activity occurs in muscles with normal and high glycogen content but not in muscles with low glycogen content. Furthermore, contraction, insulin, and glycogen content all regulate GS Ser641 phosphorylation and GS fractional activity in concert.

AMP-activated protein kinase; Akt substrate of 160 kDa; protein kinase B; TBC1D1

GLUCOSE UPTAKE and glycogen synthase (GS) are the rate-limiting steps in the regulation of glucose storage into glycogen in skeletal muscle (5, 19, 41). Glucose uptake and GS activation are stimulated by both insulin and exercise, but through distinct signaling mechanisms. Both insulin and contraction stimulate glucose transport by promoting GLUT4 translocation to the cell surface (10, 21). Insulin stimulates GLUT4 translocation through a signaling pathway that involves phosphoinositide 3-kinase (PI3-kinase) and protein kinase B (PKB, also known as Akt) (32, 65). Recent studies suggest that Akt substrate of 160 kDa (AS160; also called TBC1D4a), a Rab GTPase activating protein, plays a key role in linking PKB activation to GLUT4 translocation (40, 59). Contraction stimulates glucose transport by a mechanism independent of PI3-kinase-PKB pathway (42, 65), and activation of AMP-activated protein kinase (AMPK) is thought to be involved (23, 46). AS160 and its closely related protein TBC1D1 have been shown to be phosphorylated by AMPK and have been proposed to mediate contraction-stimulated GLUT4 translocation (9, 12, 62, 64).

GS is also activated by both insulin and contraction. Regulation of GS activity is complicated, involving covalent phosphorylation at multiple sites and allosteric activation by glucose-6-phosphate (13, 30, 41). GS is activated by dephosphorylation, and in particular dephosphorylation of Ser7,10 and Ser641,645 increases enzymatic activity (1, 60, 61). GS Ser641,645 are mainly phosphorylated by glycogen synthase kinase 3 (GSK3), and inhibition of GSK3β activity by Ser9 phosphorylation via PKB is required for insulin-mediated GS dephosphorylation and activation (6, 44). Although contraction also dephosphorylates GS at Ser641 and Ser645 (39, 44, 53, 54), recent genetic studies demonstrated that contraction-mediated activation of GS requires the glycogen-targeting subunit (RGLo) of protein phosphatase-1 (2) but not phosphorylation of GSK3 (44).

Insulin and contraction have been shown to additively increase muscle glucose uptake (22, 24, 27), but not all studies showed this additive effect (18, 21, 67). Interestingly, Zorzano et al. (67) reported that exercise enhanced insulin-stimulated glucose uptake in hindquarters of fed rats but not in hindquarters of fasted rats. A number of studies have reported that glycogen content regulates insulin- or contraction-stimulated glucose uptake (16, 17, 29, 35, 36). However, the influence of glycogen content on the additive effect of contraction and insulin on glucose uptake has not been established.

Insulin- and contraction-mediated GS activation are strongly influenced by glycogen content (14, 29, 39, 47). Glycogen seems, like contraction, to regulate GS phosphorylation independent of GSK3 (29, 39). The nine phosphorylation sites at Ser9 of GS are phosphorylated by AMPK and have been proposed to mediate contraction-stimulated GS activation. In the present study, we hypothesized that muscle glycogen content influences the additive effect of insulin and contraction on glucose uptake and GS activation. The first aim of the present study was to investigate whether contraction and insulin have an additive effect on glucose...
uptake in muscles with different glycogen contents, and to investigate the effect of glycogen content on phosphorylation of AMPK, PKB, AS160/TBC1D1, and GSK3 in muscles exposed to contraction and insulin. The second aim was to investigate the relationship between GS phosphorylation and activity when muscles with different glycogen contents were exposed to contraction and insulin alone or in combination. Specifically, GS Ser\(^2\), Ser\(^7,10\), Ser\(^641\), Ser\(^641,645\), and Ser\(^653,657\) phosphorylation were related to activity during physiological activation.

**MATERIALS AND METHODS**

**Chemicals and antibodies.** Insulin (Actrapid) was from Novo Nordisk ( Bagsværd, Denmark). Amyloglucosidase was from Boehringer Mannheim (Indianapolis, IN). 2-Deoxy-d-[\(1,2,3\H\)]glucose (29.7 Ci/mmol), d-[\(1,3\H\)]mannitol (51.0 Ci/mmol), and uridine diphosphate (UDP-[\(1\H\)]glucose (302 mCi/mmol) were from PerkinElmer Life and Analytical Sciences (Waltham, MA). Anti-GLUT4 was a gift from David E. James (Garvan Institute of Medical Research, Sydney, Australia). Anti-phospho-GS Ser\(^7,10\) was a gift from Jørgen F. P. Wojtaszewski (University of Copenhagen, Copenhagen, Denmark). Anti-phospho-GS Ser\(^641,645\) was a gift from Keki Sakamoto (University of Dundee). Anti-GS was a gift from Olu D. Pedersen (Copenhagen, Denmark). Anti-phospho-GS Ser\(^2\) and total anti-5'-AMP-activated protein kinase α2 (AMPKα2) were gifts from Grahame Hardie (University of Dundee). Anti-phospho-GS Ser\(^645,649,653,657\) was from Oncogene (San Diego, CA). Anti-phospho-GS Ser\(^641\), anti-phospho-GSK3α/β Ser\(^2\)/Ser\(^4\), anti-phospho-AMPKα Thr\(^172\), anti-phospho-acetyl-CoA carboxylase (ACC) Ser\(^79\) (equivalent of ACCβ Ser\(^218\) in rat skeletal muscle), anti-phospho-PKB Ser\(^473\), anti-phospho-PKB Thr\(^308\), anti-phospho-Akt substrate, and anti-rabbit horseradish peroxidase (HRP)-linked antibodies were from Cell Signaling (Beverly, MA). Anti-hexokinase II (C-14) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-goat HRP-conjugated antibody was from SouthernBiotech (Birmingham, AL). Anti-GSK3, anti-mouse HRP-conjugated, and anti-sheep HRP-conjugated antibodies were from Upstate (Lake Placid, NY). Enchanced chemiluminescence (ECL) was from Millipore (Billerica, MA). Other chemicals were standard analytical grades from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

**Animals.** All experimental procedures were approved by the National Animal Research Authority and conducted in conformity with the guidelines for live animals in Norway and the European Convention for the Protection of Vertebrate Animals Used in Experimental and Other Scientific Purposes. Male Wistar rats were obtained from B&K Universal (Nittedal, Norway) and acclimatized in our laboratory animal facilities for 6 or 7 days with free access to chow (RM1, B&K Universal) and tap water. The animal room was maintained at 21°C, with humidity ≥55% and a 12:12-h light-dark cycle (lights on from 6:00 AM to 6:00 PM). Muscle sampling was started at 10:00 AM, and all the experiments were performed between 10:00 AM and 2:00 PM. Muscle glycogen content was manipulated in vivo to acquire muscles of AMPK, PKB, AS160/TBC1D1, and GSK3 in muscles exposed to contraction and insulin. The rats with HG were fasted from 10:00 AM the day before experiments. The rats with LG were fasted from normal diet until the experiments. The rats with NG were kept on their normal diet until the experiments. On the day of the experiments, the weights of the rats were 120–150 g.

**Muscle preparation and incubation.** Rats were anesthetized with an intraperitoneal injection of ~10 mg of pentobarbital sodium (50 mg/ml). Epitrochlearis muscles were dissected out, suspended on contraction apparatuses at their resting length, and preincubated for 30–45 min in 3.3 ml of modified Krebs-Henseleit buffer (KHB), containing (in mM) 116 NaCl, 4.6 KCl, 1.16 MgSO\(_4\), 1.16 KH\(_2\)PO\(_4\), 25.4 NaHCO\(_3\), 2.5 CaCl\(_2\), 5.5 glucose, 2 sodium pyruvate, and 5 N-2-hydroxyethylpiperazine-N'=2-ethanesulfonic acid (HEPES), with 0.1% bovine serum albumin, pH 7.4. After preincubation, muscles were kept rested or contracted electrically (200-ms trains, 100 Hz, square wave pulses of 0.2-ms duration, 10 V delivered every 2 s) while incubating in KHB with or without 10 mU/ml insulin for 30 min as described previously (4). After incubation, muscles were promptly removed from the apparatuses, blotted on filter paper, frozen in liquid nitrogen, and stored at −70°C until analysis. All incubations were conducted in 30°C, and gas containing 95% O\(_2\) and 5% CO\(_2\) was bubbled continuously through the buffer in the test tubes.

**Glucose uptake.** For the measurement of glucose uptake, 0.25 µCi/ml 2-deoxy-d-[\(1,2,3\H\)]glucose and 0.1 µCi/ml [\(1,3\H\)]mannitol were added to the KHB while muscles were stimulated by contraction, insulin, insulin in combination with insulin or kept rested. Glucose uptake was then calculated from the intracellular accumulation of 2-deoxy-d-[\(1,2,3\H\)]glucose as described previously (4).

**Glycogen content.** Glycogen content was determined as glucose units analyzed fluorometrically with appropriate standard curves as described previously (3).

**Western blot.** Muscles were weighed and homogenized (1 mg wet wt: 25 µl) in ice-cold buffer containing (in mM) 50 HEPES, 150 NaCl, 10 Na\(_2\)PO\(_4\), 30 NaF, 1 Na\(_2\)VO\(_4\), 10 EDTA, and 2.5 benzamidine, with 0.5 µg/10 mg muscle of protease inhibitor cocktail (Sigma P8340), pH 7.4. The homogenates were rotated with 1% Triton X-100 at 4°C for 1 h and centrifuged (11,500 g) at 4°C for 10 min. Protein concentration in lysates was determined (Bio-Rad, DC Protein Assay, Hercules, CA). Muscle lysates were then diluted to 2 µg/µl and prepared with Laemmli buffer. For measurement of ACCβ Ser\(^218\) phosphorylation and GLUT4 protein, lysates were denatured at room temperature for 60 min. The lysates for other analyses were heated at 95°C for 5 min. Proteins (~20 µg) were separated by electrophoresis in 10% SDS-PAGE. An 8% SDS-PAGE was run for anti-GS to obtain clear band shifting reflecting phosphorylation state. Proteins were transferred from gel into polyvinylidene difluoride (PVDF) membrane at 250 mA for 1 h. Lysates were washed (3 × 10 min) in 80 mM Na\(_2\)HPO\(_4\), 20 mM NaH\(_2\)PO\(_4\), 100 mM NaCl, and 0.1% Tween 20 (PBS-T) and blocked in 0.5% bovine serum albumin, pH 7.4. The homogenates were centrifuged at 3,000 g for 30 min at 4°C. The supernatant/assay buffer was spotted on filter paper (Whatman ET-31, 1 cm × 2.5 cm), dropped into ice-cold 66% ethanol, and washed. Fractal activity was calculated as the activity at 0.17 mM glucose-6-phosphate in percentage of the activity at 12 mM glucose-6-phosphate.

**AMPKα2 activity.** AMPKα2 activity was measured as described previously (55, 58). Briefly, 50 µg of total muscle lysate protein was incubated at 4°C for 1 h on a shaking platform with 5 µl of protein G-Sepharose coupled to 2 µg of anti-AMPKα2 antibody. The immunoprecipitates were washed twice with 0.5 ml of lysis buffer contain-
Table 1. Glycogen content in epitrochlearis muscles from rats on normal diet, fasted, or fasted/refed after 30 min of contraction, insulin stimulation, or contraction + insulin stimulation

<table>
<thead>
<tr>
<th></th>
<th>LG</th>
<th>NG</th>
<th>HG</th>
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<tbody>
<tr>
<td>Basal</td>
<td>102.5 ± 5.7 (13)</td>
<td>184.6 ± 7.5 (15)</td>
<td>422.3 ± 14.2 (8)</td>
</tr>
<tr>
<td>Contraction</td>
<td>32.0 ± 2.5 (20)</td>
<td>64.6 ± 6.4 (24)</td>
<td>260.3 ± 26.7 (24)</td>
</tr>
<tr>
<td>Insulin</td>
<td>121.0 ± 4.1 (8)</td>
<td>203.4 ± 11.5 (10)</td>
<td>378.1 ± 41.2 (8)</td>
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<tr>
<td>Insulin +</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Contraction</td>
<td>40.6 ± 3.9 (22)</td>
<td>78.1 ± 7.8 (21)</td>
<td>320.6 ± 42.4 (24)</td>
</tr>
</tbody>
</table>

Data (in mmol/kg dry wt) are mean ± SE glycogen content in epitrochlearis muscles from rats on normal diet (NG), fasted (LG), or fasted/refed (HG) after 30 min of contraction, insulin stimulation, or contraction + insulin stimulation. After 45-min preincubation, some muscles were contracted for 30 min in the absence (Contraction) or presence (Insulin + Contraction) of 10 mU/ml insulin. Other muscles were kept rested and incubated for 30 min in the absence (Basal) or presence (Insulin) of 10 mU/ml insulin. Number of muscles in each group is given in parentheses. *Significantly lower than rested muscles; †significantly lower than NG and HG; ‡significantly higher than LG and NG.

Glucose uptake. Basal glucose uptake was higher in LG compared with NG and HG (LG > NG = HG, P < 0.001; Fig. 2A) and inversely correlated with glycogen content (R = −0.53, P < 0.002, n = 36; Fig. 2B). Contraction-stimulated glucose uptake also inversely correlated with glycogen content (R = −0.58, P < 0.001, n = 68; Fig. 2), and LG > NG > HG (P < 0.001). Similarly, insulin-stimulated glucose uptake inversely corre-

Fig. 1. Representative blots showing GLUT4, hexokinase II, and glycogen synthase (GS) protein expression in basal epitrochlearis muscles with low (LG), normal (NG), and high (HG) glycogen contents.

Fig. 2. Effects of contraction, insulin, and contraction + insulin on glucose uptake in epitrochlearis muscles with low, normal, and high glycogen contents. Glucose uptake was measured with tracer amount of 2-deoxy-2-[3H]glucose in resting muscles incubated without (open bars) or with (gray bars) insulin or in contracting muscles incubated without (black bars) or with (cross-hatched bars) insulin. A: glucose uptake. Values are means ± SE; n = 8–24. *Significantly different from resting muscles; †significantly different from muscles treated with contraction and insulin alone; ‡significantly different from NG and HG; ‡significantly different from LG and NG; ‡significantly different from contraction stimulation alone. B: correlations between glucose uptake and glycogen content. dw, Dry weight.

RESULTS

Muscle glycogen content and expression of GLUT4 and hexokinase II. Muscle glycogen content in muscle from rats with free access to chow (NG) was ~180 mmol/kg dw (Table 1). Fasting for 24 h (LG) decreased glycogen content by ~50% (P < 0.02). When 24-h-fasted rats were fed chow for another 24 h (HG), the glycogen content increased above 400 mmol/kg dw. Contraction decreased muscle glycogen in all groups (Table 1). Glycogen content in contracted HG remained higher than in rested NG (P < 0.001). Insulin did not significantly alter glycogen content in any of the groups. GLUT4 protein expression was increased by 50% in LG compared with NG and HG (LG: 151.7 ± 9.1, NG: 100.0 ± 1.7, HG: 96.5 ± 6.5, P < 0.001, n = 7 or 8; see Fig. 1 for representative blots). Hexokinase II protein expression was similar in all groups (LG: 98.5 ± 14.1, NG: 100.0 ± 12.2, HG: 103.7 ± 8.2, n = 7 in each group; see Fig. 1 for representative blots).

Glucose uptake. Basal glucose uptake was higher in LG compared with NG and HG (LG > NG = HG, P < 0.001; Fig. 2A) and inversely correlated with glycogen content (R = −0.53, P < 0.002, n = 36; Fig. 2B). Contraction-stimulated glucose uptake also inversely correlated with glycogen content (R = −0.58, P < 0.001, n = 68; Fig. 2), and LG > NG > HG (P < 0.001). Similarly, insulin-stimulated glucose uptake inversely corre-
lated with glycogen content ($R = -0.68$, $P < 0.001$, $n = 26$; Fig. 2), and LG > NG > HG ($P < 0.03$). Contraction and insulin additively increased glucose uptake in NG and HG but not in LG. It should be noted that the additive effect of insulin and contraction in NG and HG was only partial. In LG, insulin-stimulated glucose uptake was higher than glucose uptake with contraction ($P < 0.03$), and combination of contraction and insulin increased glucose uptake to a level similar to that with...
insulin stimulation alone (Fig. 2A). Glucose uptake was higher in LG and inversely correlated with glycogen content (R = 0.50, P < 0.001; n = 67; LG > NG > HG, P < 0.001; Fig. 2B) when contraction and insulin were combined.

**AMPK pathway.** Contraction increased activity of AMPKα2, the dominant isoform in skeletal muscle, in all groups. Contraction-stimulated AMPKα2 activity was lower in HG and higher in LG compared with NG (Fig. 3A). Insulin did not influence contraction-stimulated AMPKα2 activity in LG and HG but slightly reduced activity in NG. Consistent with the activity data, contraction increased AMPKα Thr172 phosphorylation in LG and NG but not significantly in HG (Fig. 3). Contraction increased AMPKα Thr172 phosphorylation more in LG than in NG (Fig. 3B). However, contraction increased ACCβ Ser218 phosphorylation to similar levels in all groups (Fig. 3C). Insulin did not alter contraction-mediated AMPKα Thr172 and ACCβ Ser218 phosphorylation.

**PKB pathway.** PKB phosphorylation was undetectable in rested and contracted muscles in the absence of insulin (Fig. 4). Insulin-stimulated PKB phosphorylation at both Ser473 and Thr308 was ~90% higher in LG than in NG. In HG, insulin-stimulated PKB phosphorylation at Thr308 was similar to NG, but PKB Ser473 phosphorylation was slightly reduced in HG (Fig. 4). Contraction decreased insulin-stimulated PKB phosphorylation at both sites in LG and NG (~20–30%), whereas contraction did not reduce insulin-stimulated PKB phosphorylation in HG (Fig. 4).

GSK3β Ser9 phosphorylation was low in rested muscles without insulin exposure (Fig. 4). Insulin increased GSK3β Ser9 phosphorylation in all groups. Insulin-stimulated GSK3β Ser9 phosphorylation was slightly higher in NG than in LG and HG (Fig. 4C). Contraction increased GSK3β Ser9 phosphorylation in NG and NG not significantly in LG (P = 0.055, Fig. 4C). GSK3β Ser9 phosphorylation was lower in contracted LG than in contracted NG and HG (LG < NG = HG; Fig. 4C). Although both contraction and insulin increased GSK3β Ser9 phosphorylation, contraction reduced insulin-stimulated GSK3β Ser9 phosphorylation in LG and HG (~25–35%, Fig. 4C). GSK3β Ser9 phosphorylation was lower in LG than in NG and HG (LG < NG = HG; Fig. 4C) when contraction and insulin were combined.

**Phospho (Ser/Thr)-Akt substrate at 160 kDa.** We used the phospho (Ser/Thr)-Akt substrate (PAS-160) antibody from Cell Signaling and got several bands (Fig. 5), of which the band at ~160 kDa (PAS-160) is thought to be AS160 and/or TBC1D1 (20, 62). Contraction increased PAS-160 phosphorylation in NG and HG, but not significantly in LG (Fig. 5A). Insulin increased PAS-160 phosphorylation to equal levels in all groups. Contraction and insulin additively increased PAS-160 phosphorylation in NG and HG but not in LG (Fig. 5A). In the present study, glucose uptake and PAS-160 phosphorylation were measured in 12 groups. Group means of PAS-160 phosphorylation did not significantly correlate with group means of glucose uptake (R = 0.32, P = 0.31; Fig. 5B).

**GS fractional activity and phosphorylation.** Basal GS fractional activity was higher in LG than in NG and lower in HG than in NG (Fig. 6A). Contraction increased GS fractional activity in all groups. In contracted muscles GS fractional activity was higher in LG and lower in HG compared with NG (Fig. 6A). Insulin increased GS fractional activity in LG and NG but not in HG. Insulin-stimulated GS fractional activity was higher in LG than in NG, whereas Ser441,645 and...
Ser641,645 phosphorylation was lower than in NG (Fig. 6).

Contraction decreased GS Ser641, Ser641,645, and Ser645,649,653,657 phosphorylation in all groups (Fig. 6, B–D). After contraction, phosphorylation of GS Ser641, Ser641,645, and Ser645,649,653,657 was higher in HG than in NG and LG. Contraction increased GS Ser7 phosphorylation in all groups (Fig. 6E) despite increasing GS fractional activity. Contraction-mediated GS Ser7 phosphorylation was lower in LG than in NG and HG. Insulin did not change GS Ser7 phosphorylation. Interestingly, GS Ser7,10 phosphorylation was decreased in NG and remained low in LG (but not significantly reduced) after contraction (Fig. 6F). In contrast, GS Ser7,10 phosphorylation increased in HG after contraction. In contracted muscles, GS Ser7,10 phosphorylation was lower in LG than in NG and higher in HG than in NG. Insulin did not influence GS Ser7,10 phosphorylation in any groups but prevented the increase in GS Ser7,10 phosphorylation in HG after contraction. Insulin decreased GS Ser641, Ser641,645, and Ser645,649,653,657 phosphorylation in all groups (Fig. 6, B–D). Insulin-mediated GS Ser641 phosphorylation was lower in LG than in NG and higher in HG than in NG (Fig. 6B). Insulin-mediated GS Ser641,645 phosphorylation was lower in LG than in NG but similar in NG and HG (Fig. 6C). During insulin stimulation GS Ser641,645,649,653,657 phosphorylation was lower in both LG and HG compared with NG (Fig. 6D).

Contraction and insulin additively decreased GS Ser641, Ser641,645, and Ser645,649,653,657 phosphorylation only in HG (Fig. 6). It should be noted that contraction alone decreased phosphorylation of these sites to a low level in LG and NG. When two stimuli were combined, GS Ser641 phosphorylation remained higher in HG than in NG and LG (Fig. 6B), whereas Ser641,645 phosphorylation was similar in all groups (Fig. 6C). GS Ser641,645,649,653,657 phosphorylation was lower in LG than in NG and HG (Fig. 6D) when insulin and contraction were combined. Data from Western blot did not show significant differences in GS expression in muscles with different glycogen contents (LG: 107.3 ± 7.8, NG: 100.0 ± 8.3, HG: 87.8 ± 6.2, n = 7 in each group; see Fig. 1 for representative blots).

**Correlations between GS fractional activity and phosphorylation.** GS fractional activity and GS phosphorylation were analyzed in 12 groups, and correlation analysis was performed on group means. GS fractional activity was inversely correlated with GS phosphorylation at Ser641 ($R = -0.94, P < 0.001$; Fig. 6H), Ser641,645 ($R = -0.81, P < 0.003$; Fig. 6f), Ser645,649,653,657 ($R = -0.75, P < 0.006$; Fig. 6J), and Ser7,10 ($R = -0.89, P < 0.001$; Fig. 6L). No significant correlation was found between GS Ser7 phosphorylation and fractional activity ($R = -0.29, P = 0.360$; Fig. 6K).

**DISCUSSION**

In the present study, insulin-stimulated glucose uptake was higher in muscles with low glycogen content compared with muscles with normal or high glycogen content, which agrees with previous studies from our group (28, 29) and other groups (16, 35, 36). Contraction-stimulated glucose uptake also inversely correlated with glycogen content, agreeing with studies in which glycogen content was manipulated by exercise and diet (17, 25). The rats with low glycogen content were fasted for 24 h and GLUT4 protein expression was increased by 50% compared with NG as we have seen before (29), and the elevated GLUT4 content may explain the higher glucose uptake in LG (24, 29). Insulin-stimulated PKB phosphorylation and contraction-mediated AMPK activity were higher in muscles with low glycogen compared with normal and high glycogen. However, although both findings agree with previous studies (15, 16, 29, 36), the downstream targets of PKB (PAS-160 and GSK3β) and AMPK (ACCβ and PAS-160) were not increased accordingly (discussed below). Furthermore, we cannot exclude that the protocol used to manipulate glycogen content (fasting and refeeding) modulates glucose uptake and signaling via mechanisms independently of glycogen content.

The present study focused on the underlying mechanism for the additive effect of contraction and insulin, and we found that contraction and insulin additively stimulated glucose uptake in NG and HG but not in LG. Still, glucose uptake was higher in LG than in NG and HG when contraction and insulin stimulation occurred simultaneously. A number of studies have previously reported that contraction and insulin additively stimulate glucose uptake (22, 24, 27). Mostly, however, the additive effect of contraction and insulin on glucose uptake is not fully additive (4, 8, 23, 53, 65), and contraction only increased insulin-stimulated glucose uptake by 25–35% in NG and HG. Some studies did not find this additivity at all (18, 21, 67). Interestingly, when contraction and insulin were combined, blockade of the insulin signaling pathway by wortmannin reduced glucose uptake to the level obtained by contraction alone (65). This could be used as an argument supporting the idea that insulin action is reduced in contracting muscles. Indeed, insulin-stimulated PKB phosphorylation was slightly reduced in NG and HG, but contraction did not influence PKB phosphorylation in HG, where combination of contraction and insulin still only showed a partial additive effect on glucose uptake.

Insulin and contraction did not have an additive effect on glucose uptake in LG. However, low glycogen nearly doubled contraction- as well as insulin-stimulated glucose uptake, and it is possible that the maximal rate of glucose uptake can be achieved by insulin alone in LG (insulin-stimulated glucose uptake was slightly higher than contraction-stimulated glucose uptake). Zorzano et al. (67) also reported that exercise and insulin additively stimulated glucose uptake in hindquarters with normal glycogen but not in hindquarters from fasted rats. In soleus, several studies did not find an additive effect of contraction and insulin on glucose uptake (4, 8, 53). Glucose uptake in soleus is also higher during insulin stimulation than during contraction (4, 8, 24, 33, 53), as occurred in epitrochlearis with low glycogen. Interestingly, glycogen content in soleus muscles is lower than in fast-twitch muscles (4, 7, 53) and rather similar to epitrochlearis muscles with low glycogen (38). It is also important to note that glucose uptake can be regulated at the site of transport as well as at the site of phosphorylation. Indeed, overexpression of hexokinase II has been reported to increase insulin-stimulated glucose uptake in skeletal muscles (11), and it is possible that glucose phosphorylation becomes a rate-limiting step in LG, where GLUT4 was upregulated without accompanying increase in hexokinase II expression. Furthermore, this may imply that the maximal rate of glucose phosphorylation, and therefore glucose uptake, occurs during insulin stimulation in LG, and therefore explains why contraction does not increase glucose uptake further.
Contraction-stimulated AMPK and insulin-stimulated PKB activation are involved in regulation of glucose uptake, but the enhanced activations of AMPK and PKB in LG were not paralleled with increased phosphorylation of their downstream substrates (ACC, PAS-160, and GSK3). Such asymmetry in insulin-mediated PKB to GSK3 phosphorylation (29, 38) and contraction-mediated AMPK to ACC has been reported previously (39, 45, 66), and it has been suggested that a small increase in PKB or AMPK activation is sufficient to induce normal phosphorylation in the downstream substrates (26, 45,
McBride et al. (43) have elegantly shown that purified glycogen and oligosaccharides with α-1,6 branch points inhibit AMPK activity in vitro, which may explain the inverse relationship between glycogen content and contraction-mediated AMPK activation. It is less obvious why PAS-160 phosphorylation does not increase in parallel with AMPK activation, but it has been shown that PAS-160 phosphorylation decreases gradually during continually AMPK activation (20).

The signaling mechanism for insulin and contraction differs at the early steps (42) but was suggested to converge at AS160 (37, 56). However, it has recently been reported that skeletal muscles express more TBC1D1 than AS160 (12, 62), and the PAS antibody recognizes phosphorylation of both proteins (20, 48, 62). Indeed, AS160 has a slightly higher molecular mass (160 kDa) than TBC1D1 (150 kDa), but we only resolved a single band at ~160 kDa (Fig. 5) as reported in most studies (37, 62, 64). Therefore, our data on PAS-160 most likely represent a combination of AS160 and TBC1D1 phosphorylation. The additive effect of insulin and contraction on PAS-160 phosphorylation in NG and HG agrees with previous data (37) and the additive effect on glucose uptake. However, the overall phosphorylation of PAS-160 did poorly reflect glucose uptake. In particular, after 30 min of contraction PAS-160 phosphorylation was not significantly increased in LG despite the fact that contraction-stimulated glucose uptake was higher than in NG. These data do not exclude AS160/TBC1D1 as important regulators of glucose uptake because the PAS antibody mainly recognizes AS160 Thr⁵⁵² and TBC1D1 Thr⁵⁰⁰ phosphorylation (48, 52), whereas both proteins are regulated by phosphorylation at other sites.

GS activity was regulated by insulin, contraction, and glycogen content as expected (30, 51). Furthermore, contraction and insulin additively increased GS fractional activity in NG and HG, whereas the two stimuli (alone or in combination) increased GS fractional activity to the same high level in LG. This pattern is rather similar to the response of glucose uptake except that contraction is a more powerful activator of GS than insulin in NG and HG. The additive effect may occur because insulin and contraction activate GS via different mechanisms (2, 44).

In the present study, contraction, insulin, and glycogen content all modulated GS Ser⁶⁴¹ phosphorylation in concert with GS fractional activity. GS phosphorylation and fractional activity were studied in 12 groups, and the quite large variations in GS fractional activities and phosphorylation make it possible to correlate phosphorylation to activity. Interestingly, GS fractional activity strongly correlates with GS Ser⁶⁴¹ phosphorylation. Although a correlation never proves casual relationship, our data suggest that GS Ser⁶⁴¹ is an important regulator of GS activity in physiological conditions. Moreover, our data also suggest that GS Ser⁶⁴¹ phosphorylation is a useful parameter to describe GS activity.

GS fractional activity also correlated with GS Ser⁶⁴¹,⁶⁴⁵ and Ser⁶⁴⁵,⁶⁴⁹,⁶⁵³,⁶⁵⁷ phosphorylation, as reported previously in human and rat skeletal muscles (38, 49). In HG, however, GS fractional activity was much lower than expected from the GS Ser⁶⁴¹,⁶⁴⁵ and Ser⁶⁴⁵,⁶⁴⁹,⁶⁵³,⁶⁵⁷ phosphorylation. Jørgensen et al. (34) reported previously that GS Ser⁶⁴¹,⁶⁴⁵ phosphorylation was similar in LG and HG, as we found in the present study. The insulin-mediated reduction in GS Ser⁶⁴⁵,⁶⁴⁹,⁶⁵³,⁶⁵⁷ phosphorylation in HG without increase in fractional activity agrees with our previous study (29). It is not obvious why these antibodies show relatively low binding to GS in muscles with high glycogen content, but GS seems to remain highly phosphorylated in HG during insulin stimulation as evaluated from gel shift (Fig. 6G) (29). Therefore, our data suggest that GS Ser⁶⁴¹ phosphorylation describes GS activity better in HG than data achieved with the antibodies against GS Ser⁶⁴¹ or Ser⁶⁴⁵,⁶⁴⁹,⁶⁵³,⁶⁵⁷.

Contraction increased GS Ser⁷ phosphorylation, as we reported previously (39). In contrast, contraction reduced GS Ser⁷,¹⁰ phosphorylation in NG, and GS Ser⁷,¹⁰ phosphorylation was even lower in contracted LG (but not significantly decreased). The antibody against GS Ser⁷,¹⁰ only binds when both sites are phosphorylated (26), which suggests that phosphorylation of GS Ser⁷ is reduced by contraction. Prats et al. (49) recently reported that GS Ser⁷,¹⁰ phosphorylation inversely correlated with GS fractional activity in skeletal muscles, and our data support this finding. Studies with mutated GS have shown that GS Ser⁷,¹⁰ phosphorylation regulates GS activity (60, 61), particularly in combination with dephosphorylation of GS Ser⁶⁴¹,⁶⁴⁵, and our data support a physiological role of these sites in regulation of GS activity. GS Ser⁷ and GS Ser¹⁰ are phosphorylated in a hierarchical manner, and it has been suggested that casein kinase 1 inevitably phosphorylates GS Ser¹⁰ upon phosphorylation of Ser⁷ (50). However, the data in the present study, which are in agreement with Jørgensen et al. (34), suggest that casein kinase 1 activity is regulated in skeletal muscles by contraction. Alternatively, protein phosphatase-1 activity may override casein kinase 1-mediated GS Ser¹⁰ phosphorylation but not contraction-mediated GS Ser⁷ phosphorylation.

Insulin and contraction activate GS by different mechanisms, and studies using genetic approaches have shown that insulin-stimulated GS activation is mediated via GSK3 (44), whereas RGL is required for contraction-mediated GS activation (2). The fact that insulin only decreased phosphorylation of the COOH-terminal sites phosphorylated by GSK3 supports...
this idea. The mechanisms for GS activation during muscle contraction seem more complex. Although contraction decreased the doublet phosphorylation of GS Ser^7, we also found that GS Ser^7 phosphorylation increased. However, the fact that contraction dephosphorylates NH_2-terminal as well as COOH-terminal sites may support the idea that protein phosphatase-1 is activated.

In conclusion, contraction and insulin additively stimulated glucose uptake in muscles with normal and high glycogen content. But not in muscles with low glycogen content. Despite the fact that contraction-stimulated AMPK activity and insulin-stimulated PKB phosphorylation were highest in LG, PAS-160 phosphorylation was not increased accordingly, which indicates that the activation of these key signaling molecules does not easily explain the rate of glucose uptake. Instead, 24-h fasting increased GLUT4 expression, which may contribute to the increased glucose uptake during contraction and insulin stimulation. Contraction- and insulin-mediated GS activation were also influenced by glycogen content; GS activity was highest in LG during all stimuli. When muscles with different glycogen contents were exposed to insulin and contraction alone or in combination, GS Ser^441 phosphorylation and fractional activity were regulated in concert, which suggests that phosphorylation of this site is important for physiological regulation of GS activity.

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