Muscle glycogen content affects insulin-stimulated glucose transport and protein kinase B activity

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Received 15 March 2000; accepted in final form 31 May 2000

Derave, W., B. F. Hansen, S. Lund, S. Kristiansen, and E. A. Richter. Muscle glycogen content affects insulin-stimulated glucose transport and protein kinase B activity. Am J Physiol Endocrinol Metab 279: E947–E955, 2000.—We investigated the possible regulatory role of glycogen in insulin-stimulated glucose transport and insulin signaling in skeletal muscle. Rats were preconditioned to obtain low (LG), normal, or high (HG) muscle glycogen content, and perfused isolated hindlimbs were exposed to 0, 100, or 10,000 µU/ml insulin. In the fast-twitch white gastrocnemius, insulin-stimulated glucose transport was significantly higher in LG compared with HG. This difference was less pronounced in the mixed-fiber red gastrocnemius and was absent in the slow-twitch soleus. In the white gastrocnemius, insulin activation of insulin receptor tyrosine kinase and phosphoinositide 3-kinase was unaffected by glycogen levels, whereas protein kinase B activity was significantly higher in LG compared with HG. In additional incubation experiments on fast-twitch epitrochlearis muscles, insulin-stimulated cell surface GLUT-4 content was significantly higher in LG compared with HG. The data indicate that, in fast-twitch muscle, the effect of insulin on glucose transport and cell surface GLUT-4 content is modulated by glycogen content, which does not involve initial but possibly more downstream signaling events.

GLUT-4; skeletal muscle; fiber type; phosphoinositide 3-kinase

GLUCOSE UPTAKE in skeletal muscle is the major site of whole body insulin-mediated glucose disposal (3). Recent evidence points to glucose transport, rather than glucose phosphorylation or glycogen synthase activity, as the rate-controlling step in insulin-induced muscle glucose utilization and glycogen synthesis in healthy and diabetic humans (8). The mechanism for insulin stimulation of muscle glucose transport involves a mobilization process through exocytosis of specific glucose transporters (GLUT-4) from intracellular storage sites to the cell surface, resulting in a net increase in active GLUT-4 transporters and glucose transport rate across the membrane (9,32). In muscles of patients with peripheral insulin resistance, GLUT-4 translocation in response to insulin is impaired (50), probably due to a defect in the insulin signaling pathway involved in glucose transport stimulation (4,14). It is, therefore, not surprising that the interest in and the search for the molecules involved in insulin signaling leading to GLUT-4 translocation in muscle have rapidly grown in recent years. Although the complete link between the initial binding of insulin with its receptor and the final mobilization of GLUT-4-containing vesicles is presently unknown, a clear picture is now emerging of the initial events in insulin signaling (reviewed in Refs. 18 and 38). In short, the binding of insulin with the extracellular part of the receptor induces insulin receptor tyrosine kinase (IRTK) activation through auto-phosphorylation at the intracellular part of the receptor. Subsequent activation of insulin receptor substrate (IRS) and its binding with phosphoinositide 3-kinase (PI3K) has been shown to be essential for the stimulation of glucose transport by insulin in muscle (18,47). The main catalytic lipid product of PI3K, phosphatidylinositol 3,4,5-trisphosphate, is believed to act as a second messenger and to activate a downstream serine-threonine protein kinase pathway involving phosphoinositide-dependent kinase-1 (PDK-1), protein kinase B (PKB; also called Akt), and protein kinase C (PKC; see Ref. 1). However, the participation of the latter kinases in the stimulation of glucose transport in insulin-stimulated muscle is still debated (21,27,44).

The aim of the present paper is to address the potential role of muscle glycogen in the regulation of insulin-stimulated glucose transport and insulin signaling in muscle. This idea originated from the frequently observed negative correlations between muscle glycogen content and insulin sensitivity. Jensen et al. (19) have elegantly shown that, in muscles where the glycogen content was altered by applying various fasting-refeeding protocols, the maximal insulin-stimulated glucose transport rate in incubated epitrochlearis muscles was inversely related to the glycogen content of the muscle. A similar observation was recently published by Kawanaka et al. (20) using an exercise-refeeding protocol. The authors suggest that the impaired insulin-induced glucose uptake during glygen...
supercompensation can be attributed to an attenuation of the amount of GLUT-4 transporters translocated to the surface membrane. In the search for the mechanism of increased insulin sensitivity after exercise, as originally observed by Richter and colleagues (34, 35), glycogen has also been proposed as a possible regulator of muscle insulin action. As illustrated with the one-leg exercise model in humans, the increased insulin sensitivity after exercise is restricted to the exercised leg, pointing to a local factor (36). Therefore, an evident candidate for this local factor could be glycogen depletion. Nevertheless, an isolated effect of glycogen content on the insulin signaling cascade in skeletal muscle has not been elucidated.

In the present study, we therefore investigated the insulin activation of glucose transport, cell surface GLUT-4 content, and activities of the insulin signaling intermediates IRTK, IRS-1-associated PI3K, and GLUT-4 content, and activities of the insulin signaling. Nevertheless, an isolated effect of glycogen content on the insulin signaling cascade in skeletal muscle has not been elucidated.

In the present study, we therefore investigated the insulin activation of glucose transport, cell surface GLUT-4 content, and activities of the insulin signaling intermediates IRTK, IRS-1-associated PI3K, and PKB-α in muscles with varying glycogen levels induced by carbohydrate deprivation or supplementation after exercise model in humans, the increased insulin sensitivity after exercise is restricted to the exercised leg, pointing to a local factor (36). Therefore, an evident candidate for this local factor could be glycogen depletion. Nevertheless, an isolated effect of glycogen content on the insulin signaling cascade in skeletal muscle has not been elucidated.

In the present study, we therefore investigated the insulin activation of glucose transport, cell surface GLUT-4 content, and activities of the insulin signaling intermediates IRTK, IRS-1-associated PI3K, and PKB-α in muscles with varying glycogen levels induced by carbohydrate deprivation or supplementation after a swimming exercise bout. The use of the isolated perfused rat hindquarter model allows us to study these effects simultaneously in four calf muscles with different muscle fiber type composition.

**METHODS**

**Experimental animals.** All experiments were approved by the Danish Animal Experiments Inspectorate and complied with the Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23, revised 1985). Male Wistar rats (mean weight ± SE was 87 ± 2 g, n = 66) were preconditioned to obtain three different subgroups with varying muscle glycogen concentrations, as described previously (17). Rats were subjected to 2 h of swimming in water maintained at 32–35°C with weights (5% of body weight) attached to their tails. In the 24 h preceding the swim, their food intake was restricted to 4 g (~60% of normal intake). After swimming, all rats had free access to tap water, and they were fed ad libitum with either lard (low glycogen, LG), with 4 g of normal rat chow and lard ad libitum (normal glycogen, NG), or with normal rat chow and a 20% glucose drinking solution (high glycogen, HG) until 3–6 h before perfusions or incubations. Rat muscles were perfused or incubated between 18 and 24 h after the swimming bout.

**Hindlimb perfusions.** The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Surgery was performed as described by Ruderman et al. (37) for isolated hindquarter perfusion. Rats were killed by intracardiac pentobarbital sodium injection. All perfusions lasted 25 min using a constant flow of 6 ml/min, perfusing both legs. The perfusion medium (100 ml) was constantly gassed with a mixture of 95% oxygen-5% carbon dioxide. At the onset of the perfusion, the first 10 ml of the perfusion medium that passed through the hindquarter were discarded, and thereafter the remaining 90 ml were recirculated. During the initial 20 min of perfusions, a cell-free and glucose-free perfusate was used, consisting of Krebs-Ringer bicarbonate buffer solution (KRB), 4% BSA (fraction V; Sigma Chemical), 0.15 mM pyruvate, and 4.2 IU/ml heparin, as described previously (49). To measure the muscle glucose transport during the last 5 min of perfusion, the hindquarters were exposed to a new medium, similar to the previous medium but with the following additions: 8 mM 2-deoxy-D-glucose and 1 mM mannitol together with radioactive labeled tracers 2-deoxy-D-[2,6,3H]glucose (specific activity 51 Ci/mmol; Amersham International) and [1-3H]mannitol (specific activity 57 mCi/mmol; Amersham International), yielding an activity of 0.075 and 0.05 μCi/ml, respectively, as previously described (49). Throughout the whole perfusion period, the perfusion media contained either 0, 100, or 10,000 μU/ml insulin (Actrapid; Novo Nordisk), so there were nine experimental groups: basal condition and submaximal and maximal insulin stimulation in rats with HG, NG, and LG. The perfusion pressure ranged between 40 and 60 mmHg. At the end of the experiment, circulation was stopped, and, from both legs, muscle samples were taken from four different parts of the calf musculature, representing a whole range of fiber type distributions. The respective proportions of slow-twitch oxidative, fast-twitch oxidative glycolytic, and fast-twitch glycolytic fibers of young rats (60–100 g) are taken from Maltin et al. (31) and are given in parentheses. The white, most superficial part of the gastrocnemius (0:20:80), the plantaris (10:50:40), the red, deep proximal and medial portion of gastrocnemius (10:55:35), and the soleus (55:40:5) were trimmed of connective tissue, blotted, and freeze-clamped with aluminum clamps cooled in liquid nitrogen. The biopsies were stored at −80°C until analyzed.

**Glycogen and glucose transport measurements.** Muscle glycogen content was measured as glucose residues by a hexokinase method after acid hydrolysis (28). The 2-deoxyglucose uptake (μmol·g−1·h−1) by the different muscles was determined in perchloric acid extracts as described previously (49).

**Photoaffinity labeling of cell surface GLUT-4.** On a subgroup of animals of the HG and LG groups, epitrochlearis muscles were stimulated with insulin during in vitro incubations and subsequently exposed to Bio-LC-ATB-BMPA, synthesized as described previously (24), for surface labeling of GLUT-4. Muscle incubation was chosen for this purpose because the high production costs of this compound do not allow surface labeling experiments in the perfused hindlimb. The epitrochlearis muscle was chosen because it is a fast-twitch muscle containing approximately 75% fast-twitch glycolytic fibers (43) and because it is more suitable for incubation than the fast-twitch muscles of the hindlimb. Rats were anesthetized as described above, and the whole body was perfused (flow ~20 ml/min) for 1 min through the left ventricle with KRB containing 8 mM glucose, 1 mM pyruvate, and 0.2% BSA. The epitrochlearis muscles were carefully dissected out and incubated in KRB containing 8 mM glucose, 1 mM pyruvate, and 0.2% albumin for at least 30 min at 29°C with constant shaking. The incubation flasks were constantly gassed with a mixture of 95% oxygen-5% carbon dioxide. Subsequently, the muscles were transferred to a new oxygenated KRB solution containing 2 mM pyruvate, 0.2% albumin, and 0, 100, or 10,000 μU/ml insulin. After 30 min of insulin stimulation, the muscles were transferred to a dark room and incubated in 1 ml of KRB buffer containing 400 μM Bio-LC-ATB-BMPA in the presence of 0, 100, or 10,000 μU/ml insulin. After 8 min of incubation at 18°C, muscles were irradiated for 6 min in a Rayonet photochemical reactor (Southern New England Ultraviolet, Branford, CT) using 300-nm lamps. After irradiation, the two muscles from the same rat were pooled, immediately blotted, trimmed, frozen in liquid nitrogen, and stored at −80°C. Solubilized crude membranes were prepared as described previously, with slight modification (30). Briefly, muscles were homogenized in ice-cold 20 mM HEPES, 5 mM NaEDTA, and 255 mM sucrose (HES buffer; pH 7.2) and later centrifuged at 320,000 g for 60 min. The pellet was resuspended and solubilized in the HES-buffer containing 2% (wt/vol) Thesit. The homoge-
nate was rotated for 1 h at 4°C and then centrifuged at 120,000 g for 30 min. Solubilized crude membranes (150 µg) in 500 µl HES buffer containing 1% (wt/vol) Thesit were then mixed with 100 µl of a 50% slurry of immunopure immobilized streptavidin on 6% beaded agarose (Pierce Chemical, Rockford, IL). The samples were incubated overnight at 4°C, and the precipitates were washed four times in 1% Thesit-PBS buffer, four times in 0.1% Thesit-PBS buffer, and finally in PBS. The biotinylated protein was separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% nonfat milk incubated with a polyclonal anti-COOH-terminal peptide GLUT-4 antibody (29) diluted 1:4,000 (vol/vol) in Tris-buffered saline and 0.1% (vol/vol) Tween 20. Labeled proteins were visualized by the enhanced chemiluminescence method (Pierce Chemical) and were compared with a standard to correct for intergel variations. Results were expressed relative to the value of nonstimulated HG muscle (set at 1).

**IRTK activity.** IRTK activity was measured in duplicate by a modification (16) of the method described by Kleine et al. (23). Short, microtiter wells were coated with anti-insulin receptor antibody, as described previously (22), and 30 µl of muscle homogenate supernatant were added to each well. IRTK activity was estimated as incorporation of phosphate in poly-Glu-Tyr (4:1) after addition of the substrate in the presence of [32P]ATP and scanning of filters using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA; see Ref. 16). The binding capacity in each well was measured using 125I-labeled insulin.

**IRS-1-associated PI3K activity.** PI3K was immunoprecipitated as previously described, with minor modifications (46). Muscle samples (white gastrocnemius) were homogenized (OMNI 2000; Omni International, Warrenton, VA) in ice-cold solubilization buffer consisting of 50 mM HEPES (pH 7.4), 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM NaF, 10 mM Na₃VO₄, 1.5 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail tablet, and insoluble material was removed by centrifugation for 10 min. Equal amounts of protein (250 µg) were solubilized in 100 µl of a 50% slurry of immunopure immobilized streptavidin on 6% beaded agarose (Pierce Chemical, Rockford, IL). The samples were incubated overnight at 4°C, followed by protein A-Sepharose 6MB (TI 70.1; Beckman Instruments, Fullerton, CA) for 30 min. IRS-1 was immunoprecipitated with anti-IRS-1 antibody (no. 06–608; UBI), Waltham, MA for 2 h at 4°C, followed by protein A-Sepharose 6MB (4°C, overnight; Pharmacia). The precipitates were successively washed, as described previously (46), and PI3K activities were measured immediately on immunoprecipitates by in vitro phosphorylation of phosphatidylinositol. Sonicated phosphatidylinositol (20 µg phosphatidylinositol/ sample in 10 µl of 5 mM HEPES (pH 7.4)) was added to each sample. The PI3K reaction (30°C, 10 min) was started by addition of 10 µl of 50 mM MgCl₂, 250 µM ATP, and 0.5 µCl/µl γ-[32P]ATP (specific activity 5,000 Ci/mmol; Amersham International) in a buffer consisting of 20 mM HEPES (pH 7.4), 0.4 mM EGTA, and 0.4 mM Na₂PO₄ and was stopped by addition of HCl. Lipids were harvested by chloroform-methanol (1:1) extraction and were applied to a silica gel TLC plate. The plates were developed (45–60 min) in chloroform-methanol-ammonium hydroxide-water (45:35:3:7 by volume). Spots were quantified on a phosphorscreen using a PhosphorImager (Molecular Dynamics) and were compared relative to a standard sample.

**PKB activity.** Muscle samples were homogenized in 10 vol of ice-cold buffer (pH 8) containing 4 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1 µM microcystin, 0.1% 2-mercaptoethanol, and a protease inhibitor cocktail tablet, and insoluble material was removed after a 13,000-g centrifugation for 10 min (4°C). Equal amounts of protein (250 µg) were immunoprecipitated with 4 µg anti-PKB-α antibody (no. 06–608; UBI), and PKB activity was measured with the PKB immunoprecipitation kinase assay kit from UBI according to the protocol from the manufacturer except that the kinase reaction was allowed to proceed for 30 min at room temperature. The radioactivity of filter papers was quantified using a β-scintillation counter (TRICARP 1500; Packard, Meriden, CT). PKB activity is expressed as milliunits per milligram protein, where 1 unit = 1 nmol phosphate incorporated per 30 min.

**PKB phosphorylation.** Supernatants from homogenates from the PKB activity assay were used for evaluation of PKB phosphorylation. Equal amounts of protein (7.5 µg) were separated on 10% bis-Tris NuPage gels (NOVEX, San Diego, CA) with the PHOSPHOPRINT Akt (Ser473) antibody kit according to the manufacturer’s protocol. Bands were quantified by the use of the FujiFilm CCD camera and the Image Gauge software (Fuji Photo Film, Elmsford, NY). Results are expressed as percent of internal standard (PKB-α; UBI).

**Statistics.** Statistical evaluation of the data was done by unpaired t-tests to compare HG with LG (when no intermediate group was included) or one-way ANOVA to compare HG, NG, and LG, using the Student-Newman-Keul’s method for post hoc multiple comparisons where appropriate. Linear correlations were calculated with the Pearson product moment test. Data are presented as means ± SE, and the level of significance was chosen at 0.05.

**RESULTS**

**Muscle glycogen content.** Feeding the rats different diets after the swimming exercise bout resulted in markedly different muscle glycogen levels at the time the experiments were performed, i.e., 18–24 h after swimming (Fig. 1). In the NG group that received a mixed carbohydrate/fat diet, glycogen levels had returned to preexercise levels (45–50 µmol/g wet wt in plantaris and white and red gastrocnemius and 17 µmol/g in the soleus). The rats of the HG group that received a carbohydrate-rich diet were glycogen supercompensated, with calf muscle glycogen levels 2.0- to 3.6-fold higher (P < 0.05 for all muscles investigated) compared with the NG group (Fig. 1). The rats of the LG group were deprived of carbohydrates and were supercompensated, with high HG, normal NG, or low LG muscle glycogen contents. Data are pooled from rats that were perfused with 100 and 10,000 µU/ml insulin. Data are presented as means ± SE for 13–14 measurements. ww, Wet wt. *P < 0.05, significant difference compared with NG.
still glycogen depleted at the time of experiments, with values being 1.8- to 2.6-fold lower \( (P < 0.05) \) for all muscles investigated) compared with the NG group (Fig. 1). In the surface-labeling experiments, epitrochlearis muscle glycogen content was not measured due to lack of tissue. In previous experiments with an identical protocol, we had observed a threefold higher epitrochlearis glycogen content in HG compared with LG rats.

**Glucose transport.** Basal muscle glucose transport rate, estimated from the uptake of isotopic 2-deoxyglucose, was significantly higher \( (P < 0.05) \) in LG compared with HG and NG in the white and red gastrocnemius but not in the plantaris and soleus (Fig. 2). Stimulation with a submaximal insulin concentration \( (100 \mu U/ml) \) in muscles with NG caused an approximately half-maximal stimulation of 2-deoxyglucose uptake in the four muscle types. At a maximally stimulating \( (35) \) insulin concentration \( (10,000 \mu U/ml) \) 2-deoxyglucose uptake in NG rats was higher in the oxidative than in the glycolytic muscles \( (soleus > red \text{ gastrocnemius} > \text{plantaris} > \text{white \text{ gastrocnemius}}) \). With regard to the different muscle glycogen levels, there appear to be major differences both in insulin sensitivity and in responsiveness. In white and red gastrocnemius, plantaris, and soleus, respectively, 2-deoxyglucose uptake at submaximal insulin stimulation was 4.6-, 3.2-, 2.6-, and 1.6-fold higher \( (P < 0.05) \), except for the soleus in LG compared with HG rats (Fig. 2). When correcting for the different basal glucose transport values, the respective increases in 2-deoxyglucose uptake from basal to submaximal insulin stimulation were 2.4, 9.4, 6.5, and 10.2 \( \mu \text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \) in glyco-gen-supercompensated muscles (HG) compared with 15.2, 30.5, 17.3, and 17.3 \( \mu \text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \) in glycogen-depleted muscles (LG; \( P < 0.05 \) for all muscles except soleus). Maximal insulin-stimulated \( (10,000 \mu U/ml) \) glucose transport rates were only affected \( (P < 0.05) \) by glycogen levels in the white gastrocnemius, with the increase from basal to maximal insulin stimulation being 7.0, 12.6, and 15.0 \( \mu \text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \) in HG, NG, and LG, respectively (Fig. 2A). Whenever a significant difference in 2-deoxyglucose uptake was observed between HG and LG, the value of the NG group was intermediate to those extremes. Thus we found significant negative correlations between muscle glycogen content and glucose transport rate at submaximal (white and red gastrocnemius) and maximal insulin stimulation (white gastrocnemius), as shown in Table 1. It is noteworthy that, in glycogen-depleted rats (LG), a high physiological insulin concentration \( (100 \mu U/ml) \) caused a maximally effective increase in glucose transport, as no further increase was noticed at 10,000 \( \mu U/ml \) insulin in white and red gastrocnemius and plantaris.

**GLUT-4 cell surface content.** To measure cell surface GLUT-4 content in predominantly fast-twitch glycolytic muscle fibers, where the largest effect of glycogen content on glucose transport was observed, it was necessary to use the intact epitrochlearis muscles, since the white part of the gastrocnemius muscle could not be incubated. As described in METHODS, the epitrochlearis, like the white gastrocnemius, contains 75–80% fast-twitch glycolytic fibers. The GLUT-4 cell surface content in incubated epitrochlearis muscles in the presence of 0, 100, and 10,000 \( \mu U/ml \) insulin, respectively, was 1.8-fold, 3.5-fold \( (P < 0.05) \), and 2.3-fold \( (P < 0.05) \) higher in LG compared with HG (Fig. 3). The insulin-induced increase in GLUT-4 cell surface content in muscles with HG vs. LG, respectively, was 2.2 ± 0.4 vs. 8.6 ± 1.7 (arbitrary units) at submaximal insulin \( (P < 0.05) \) and 5.8 ± 0.8 vs. 13.4 ± 1.9 \( (P < 0.05) \) at maximal insulin concentration (Fig. 3).
Early insulin signaling activity in white gastrocnemius. Because the largest effect of glycogen content on glucose transport was observed in the white gastrocnemius, we initially decided that possible glycogen effects on insulin signaling would be largest in this muscle. Thus initial signaling assays were performed only in white gastrocnemius. Furthermore, we assayed muscles with LG and HG, and only if differences between these groups were found was NG assayed also.

Basal IRTK and PI3K activities were similar in LG and HG (Fig. 4). The IRTK activity increased \((P < 0.05)\) \(\sim 2\)-fold with submaximal and \(\sim 16\)-fold with maximal insulin stimulation in both groups. IRTK activity was not affected by the muscle glycogen content at any insulin concentration. It should be noted that the increase in IRTK activity in response to \(100 \mu\text{U/ml}\) insulin was only 5 and 3% of the increase induced by \(10,000 \mu\text{U/ml}\) in HG and LG, respectively (Fig. 4A), whereas the glucose transport rate at submaximal insulin levels was 34 and 94% of the maximal response, pointing to a large spare IRTK activity. By contrast, PI3K activity (Fig. 4B) increased \((P < 0.05)\) approximately twofold with submaximal and approximately fivefold with maximal insulin stimulation and was not affected by the muscle glycogen content at any insulin concentration.

**PKB-α phosphorylation and activity.** In the present study, we initially evaluated the degree of Akt1/PKB-α activity by the degree of Ser 473 phosphorylation. Akt/PKB phosphorylation was increased more by insulin in LG than in HG, but the difference only reached statistical significance at maximal insulin levels (Fig. 5). Because differences in Akt/PKB phosphorylation were found between LG and HG, we next studied Akt/PKB activity at all three glycogen levels and in both the white and red gastrocnemius (Fig. 6). The basal PKB activity was not different between the glycogen groups. In the white gastrocnemius, PKB activity increased significantly with submaximal and maximal insulin stimulation in all groups, but the increases were significantly affected by the muscle glycogen content. In HG, NG, and LG, respectively, submaximal insulin

![Table 1. Relationship (r values) between muscle glycogen concentration, glucose transport rate, and PKB activity in white and red gastrocnemius muscles after submaximal and maximal insulin stimulation](image)

<table>
<thead>
<tr>
<th>Glycogen × Glucose Transport</th>
<th>Glycogen × PKB Activity</th>
<th>Glucose Transport × PKB Activity</th>
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<td><strong>White Gastrocnemius</strong></td>
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<td>Insulin, (\mu\text{U/ml})</td>
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<td>100</td>
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<td>10,000</td>
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<td><strong>Red Gastrocnemius</strong></td>
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<td>Insulin, (\mu\text{U/ml})</td>
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<tr>
<td>100</td>
<td>21</td>
<td>(-0.69^†)</td>
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<td>10,000</td>
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PKB, protein kinase B; \(n\), no. of measurements. \(^*\)Significant correlation \((P < 0.05)\). \(^†\)Significant correlation \((P < 0.01)\).
stimulation caused 1.5-, 2.2-, and 3.8-fold increases (\(P < 0.05\) for LG vs. NG and HG), and maximal insulin stimulation caused 5.0-, 11.0-, and 16.7-fold increases (\(P < 0.05\) for LG vs. HG) in PKB activity. Thus PKB activity was negatively correlated with muscle glycogen content at both insulin concentrations, and PKB activity also correlated positively (\(r = 0.68; P < 0.01\)) with glucose transport rates at submaximal insulin stimulation (Table 1). In the red gastrocnemius, insulin stimulation of PKB activity was not dependent on glycogen (Fig. 6B). Interestingly, although PKB activity was not dependent on muscle glycogen in this fiber type, there still was a significant positive correlation between PKB activity and glucose transport at submaximal insulin concentrations (Table 1).

**DISCUSSION**

In the present study, we have shown that the effect of glycogen on insulin-stimulated glucose transport and GLUT-4 translocation is restricted to fast-twitch muscle fibers. In addition, we have shown that this glycogen effect on insulin action may involve downstream (Akt/ PKB) but not upstream (IRTK- and IRS-1-associated PI3K) insulin signaling events. This fiber-type specificity could not be attributed to fiber-type dependent differences in effectiveness in glycogen depletion/supercompensation protocols, since the degree of difference in glycogen content between HG and LG was not smaller in slow-twitch (6.4-fold in soleus) compared with fast-twitch (4.5-fold in white gastrocnemius) muscle. We have recently reported a similar fiber type-specific regulatory role of glycogen in contraction stimulation of glucose transport (10).

When trying to understand how glycogen, directly or indirectly, affects insulin action on glucose uptake, it is critical to know the precise site of regulation of the glucose utilization process. By using the sensitive biotinylated bis-mannose photolabel (Bio-LC-ATB-BMPA; see Ref. 24), we presently show that most, if not all, of the variation in glucose transport with varying glycogen levels can be explained by the number of GLUT-4 proteins on the surface membrane. This was also recently shown by Kawanaka et al. (20) in maximally insulin-stimulated muscles using the radioactive bis-mannose photolabel.

It thus appears that a high glycogen content in muscle is associated with a state of insulin resistance to translocate GLUT-4-containing vesicles. It was previously hypothesized that glycogen inhibits GLUT-4 translocation by an association between GLUT-4-containing vesicles and glycogen particles, making the former “unavailable” for translocation. However, we presently believe that regulation of glucose transport by glycogen is not situated at the translocation level itself but is more upstream at the signaling level. Kawanaka et al. (20) have recently shown that glycogen supercompensation inhibits not only insulin-stimulated glucose transport but also insulin-stimulated amino acid transport. It is therefore tempting to speculate that glycogen levels affect insulin signaling in muscle. Tyrosine phosphorylation of IRS-1 by IRTK activity and subsequent binding to and activation of PI3K have been identified as two important initial events in the insulin transduction pathway leading to
increased glucose transport. As shown in Fig. 4, neither IRTK activity nor IRS-1-associated PI3K activity is different between muscles with HG or LG during stimulation with 100 or 10,000 μU/ml insulin. By analogy, in vivo studies in humans (46) and rats (13) have failed to detect increased insulin-stimulated IRTK and PI3K after a glycogen-depleting exercise bout. From a teleological point of view, however, it is not completely surprising that a metabolic feedback signal (e.g., a signal indicating “intracellular energy/substrate abundance” during glycogen supercompensation) will not interfere with the initial step of insulin signaling (IRTK activity), since this step is shared by other signaling pathways of insulin, leading to nonmetabolic events (e.g., the mitogen-activated protein kinase signaling cascade leading to cell growth and mitosis). In other words, it could be expected that the “product inhibition” exerted by glycogen is restricted to processes that lead to its synthesis.

PKB (also known as Akt or Rac) has been proposed to be a mediator of the insulin signaling pathway downstream of PI3K (reviewed in Refs. 1 and 38). PKB is activated by phosphorylation on Thr308 and Ser473 by PDK-1 and phosphoinositide-dependent protein kinase-2 (PDK-2) and binding of phosphatidylinositol 3,4,5-trisphosphate, the major catalytic product of activated PI3K (38). A new finding of the present study is that PKB-α/Akt1 activation (assessed by an in vitro activity assay and by the phosphorylation status on Ser473) in response to a standardized submaximal or maximal insulin stimulus is dependent on the muscle glycogen content. It is speculative but tempting to attribute causality to the relationship between the changes in PKB activation and the parallel changes in glucose transport and cell surface GLUT-4 content in insulin-stimulated muscles with varying glycogen content. The majority of recent papers support the notion of a functional link between PKB activation and glucose transport stimulation in muscle (15, 23, 25, 26, 42), whereas others do not (27, 39). In the present study, glycogen content in the red gastrocnemius muscle affected insulin-stimulated glucose transport but not PKB activity. The data on this muscle type with mixed fiber-type composition (red gastrocnemius) therefore indicate that a causal link between the PKB activity and the glucose transport rate should be taken with precaution. Furthermore, it should be noted that not only PKB but also some isoforms of PKC have recently been suggested to be downstream effectors of PI3K and to be involved in insulin-stimulated glucose transport regulation. However, the present literature in this area is still inconclusive, because most work so far has been limited to cultured cell lines, and controversy still exists about which isoforms of PKC are actually involved. Most reports indicate the atypical isoforms ζ and λ (40) as the ones that participate in insulin stimulation of glucose transport, whereas others also report the involvement of the conventional isoform β2 (6) and the novel isoform δ (5). In relation to the present study, it will be most interesting to investigate in mammalian skeletal muscle whether the insulin activation of various PKC isoforms is dependent on the muscle glycogen content.

In a subset of experiments, the in vivo venous plasma glucose concentrations were measured before perfusion in rats from the HG and LG groups. As expected from their food intake, the results show significantly (P < 0.05; n = 10–12) higher circulating glucose levels in HG (12.4 ± 1.1 mM) compared with LG (6.3 ± 0.8 mM). Thus it might be suggested that the downregulation of insulin activation of PKB and glucose transport might in fact not be directly related to glycogen levels but rather to the hyperinsulinemia and hyperglycemia that will prevail in the carbohydrate-fed HG rats compared with the fat-fed LG rats. Such a notion is difficult to evaluate, because the different muscle glycogen levels in the present study cannot be separated from hyperinsulinemia and hyperglycemia. However, the following arguments support the notion that it is the glycogen level per se rather than hyperinsulinemia and hyperglycemia that is responsible. First, there was no effect of glycogen levels on the proximal insulin signaling steps, which might be expected if we are dealing with a downregulation of insulin signaling by chronic insulin stimulation, as recently shown by Pryor et al. (33). Second, muscles were preperfused for 20 min with a standardized glucose-free medium before measurements were made, so that, at least for the last 20 min, insulin and glucose were identical in the groups. Finally, in a previous study in which insulin resistance of glucose uptake in muscle was found in muscles with high glycogen levels (19), the glycogen manipulation protocol in some of the studies included a period of 12-h fasting before the experiment in groups, ending up with different glucose levels and different insulin effect on glucose uptake. Although plasma insulin and glucose levels in these rats were not reported, the 12-h fasting in both groups is believed to result in largely similar values. Taken together, although final proof is lacking, we believe that the evidence favors a direct role of glycogen in insulin action rather than an indirect role related to high insulin and glucose levels in the blood. A striking finding of the present study is that the differences in insulin activation of PKB with varying muscle glycogen levels did not appear to involve IRS-1-associated PI3K. Interestingly, two papers published last year (27, 39) reported observations that support ours, namely that hyperglycemia-induced insulin resistance in fast-twitch fibers coincides with impaired PKB activation but not PI3K activation. This supports the attractive hypothesis that the mechanisms by which hyperglycemia and glycogen supercompensation induce insulin resistance are similar and involve a downregulation of the insulin signaling at the site of PKB. Both situations indeed share the situation of abundant carbohydrate availability and the necessity to downregulate the entry of more glucose into the muscle cells to prevent overaccumulation. Furthermore, we show that the opposite situation, i.e., decreased carbohydrate availability, is associated with facilitated insulin stimulation of PKB. To further illus-

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trate the pivotal role of PKB in insulin action, Chen et al. (7) have shown that osmotic shock inhibits insulin-stimulated glucose transport by downregulating PKB phosphorylation without affecting IRS-1 or PI3K activity. In an attempt to explain decreased PKB activation in the face of normal PI3K activity, an alternative PI3K-independent upstream signaling pathway could be hypothesized. Interestingly, Wojtaszewski et al. (48) have reported that insulin alone can modestly activate PKB, and insulin plus exercise can markedly activate PKB in mice in which the muscle-specific insulin receptor was knocked out and in which there consequently was no apparent activation of IRTK or PI3K.

One can only speculate on how high glycogen levels, directly or through another metabolite or signal, regulate insulin activation of PKB. In this context, one might speculate why the relationship is only observed in fast-twitch glycolytic muscle. Glycogen is known to form complexes with the sarcoplasmic reticulum and the enzymes that regulate glycogen metabolism. In fast-twitch glycolytic muscle, sarcoplasmic reticulum is more abundant than in oxidative muscle (2), and it might be that some of the enzymes (e.g., PKB) of the insulin signaling cascade can also bind to the glycogen-sarcoplasmic reticulum complex, which might make them less available in the cascade. This remains to be established, however.

One important health benefit of exercise is the increased insulin sensitivity in the period after an exercise bout. The mechanism for this increased insulin sensitivity is poorly understood but could possibly be ascribed to the low muscle glycogen levels caused by exercise; hence, the mechanism involves the stimulatory role of glycogen depletion on downstream insulin signaling, as illustrated by the present results in fast-twitch rat muscle. Indeed, Thorell et al. (41) and Wojtaszewski et al. (48) reported in humans and in mice, respectively, that insulin-stimulated muscle glucose uptake was enhanced immediately after glycogen-depleting exercise, which could be associated with the higher PKB phosphorylation compared with the same insulin stimulus without prior exercise. In contrast, one study reported that 4 h after exercise, when insulin sensitivity is still markedly increased, PKB phosphorylation and activity is not different between exercised and nonexercised muscles (45). Thus some but not all recent studies suggest that increased postexercise insulin sensitivity may be caused by enhanced downstream signaling through an exercise factor that might be glycogen depletion. However, glycogen depletion is clearly not the only factor, since some studies have shown that increased insulin sensitivity persists when glycogen levels have returned to baseline levels (12, 35), and another study has indicated that the exercise-induced increase in insulin sensitivity requires a serum factor (11).

In conclusion, this study indicates that the activation of muscle glucose transport and GLUT-4 translation in response to a submaximal or maximal insulin stimulus is modulated by the glycogen content in fast-twitch but not slow-twitch skeletal muscle. Furthermore, we have shown that insulin activation of PKB-α/Akt1 but not IRTK- or IRS-1-associated PI3K activity is also modulated by muscle glycogen content in fast-twitch muscle in a similar manner. Thus inhibition and enhancement of insulin action by high and low muscle glycogen levels, respectively, is proposed to involve downstream but not initial insulin signaling events.

G. D. Holman (University of Bath, UK) is acknowledged for kind donation of the surface labeling compound. We are grateful to B. Bolmgren, E. Hornemann, and P. Jensen for superior technical assistance.

This study was supported by Danish National Research Foundation Grant 504–14, by the Novo Nordisk Foundation, and by the Danish Diabetes Research Foundation.

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