Epinephrine-stimulated glycogen breakdown activates glycogen synthase and increases insulin-stimulated glucose uptake in epitrochlearis muscles

Anders J. Kolnes,1 Jesper B. Birk,2 Einar Eilertsen,3 Jorid T. Stuenæs,3 Jørgen F. P. Wojtaszewski,2 and Jorgen Jensen2,4

1Charles University Third Faculty of Medicine, Prague, Czech Republic; 2Molecular Physiology Group, The August Krogh Centre, Department of Nutrition, Exercise and Sports, Copenhagen University, Copenhagen, Denmark; 3National Institute of Occupational Health, Oslo, Norway; and 4Department of Physical Performance, Norwegian School of Sports Sciences, Oslo, Norway

Submitted 17 June 2014; accepted in final form 1 December 2014

Kolnes AJ, Birk JB, Eilertsen E, Stuenæs JT, Wojtaszewski JF, Jensen J. Epinephrine-stimulated glycogen breakdown activates glycogen synthase and increases insulin-stimulated glucose uptake in epitrochlearis muscles. Am J Physiol Endocrinol Metab 308: E231–E240, 2015. First published December 2, 2014; doi:10.1152/ajpendo.00282.2014.—Epinephrine increases glycogen synthase (GS) phosphorylation and decreases GS activity but also stimulates glycogen breakdown, and low glycogen content normally activates GS. To test the hypothesis that glycogen content directly regulates GS phosphorylation, glycogen breakdown was stimulated in condition with decreased GS activity. Saline or epinephrine (0.02 mg/100 g rat) was injected subcutaneously in Wistar rats (~130 g) with low (24-h-fasted), normal (normal diet), and high glycogen content (fasted-refeeded), and epitrochlearis muscles were removed after 3 h and incubated ex vivo, eliminating epinephrine action. Epinephrine injection reduced glycogen content in epitrochlearis muscles with high (120.7 ± 17.8 vs. 204.6 ± 14.5 mmol/kg, P < 0.01) and normal glycogen (89.5 ± 7.6 vs. 152 ± 8.1 mmol/kg, P < 0.01), but not significantly in muscles with low glycogen (90.0 ± 5.0 vs. 102.8 ± 7.8 mmol/kg, P = 0.17). In saline-injected rats, GS phosphorylation at sites 2+2a, 3a+3b, and 1b was higher and GS activity lower in muscles with high compared with low glycogen. GS sites 2+2a and 3a+3b phosphorylation decreased and GS activity increased in muscles where epinephrine decreased glycogen content; these parameters were unchanged in epitrochlearis from fasted rats where epinephrine injection did not decrease glycogen content. Incubation with insulin decreased GS site 3a+3b phosphorylation independently of glycogen content. Insulin-stimulated glucose uptake was increased in muscles where epinephrine injection decreased glycogen content. In conclusion, epinephrine stimulates glycogenolysis in epitrochlearis muscles with normal and high, but not low, glycogen content. Epinephrine-stimulated glycogenolysis decreased GS phosphorylation and increased GS activity. These data for the first time document direct regulation of GS phosphorylation by glycogen content.

SKELETAL MUSCLE GLYCOGEN is an important energy substrate during exercise (24). In addition, skeletal muscle glycogen functions as major store for ingested carbohydrates and contributes to regulation of blood glucose (21). The glycogen content in skeletal muscles is limited, and high glycogen content inhibits glycogen synthase (GS) activity, whereas low glycogen content activates GS (9, 19, 29, 33). This inverse relationship between glycogen content and GS activation has been established by manipulating glycogen content by fasting-refeeding (19, 28–30) and exercise-diet protocols (33). Epinephrine stimulates glycogen breakdown in skeletal muscles as muscle contraction does, but epinephrine also increases GS phosphorylation and decreases GS activity (5, 18, 38). The effect of epinephrine-stimulated glycogen breakdown on GS phosphorylation after removal of epinephrine has not been carefully characterized.

GS is phosphorylated at at least nine sites, and increased phosphorylation decreases fractional activity (5, 20, 36). Epinephrine stimulates GS phosphorylation via β2-adrenergic receptors, cAMP, and activation of PKA; activated PKA phosphorylates GS at sites 2, 1a, and 1b (5, 36). In addition, PKA increases GS phosphorylation via phosphorylation of the protein phosphatase-1 (PP1) glycogen-targeting regulatory subunit RGL. Phosphorylation of RGL dissociates PPI from the glycogen particle and increases GS phosphorylation (36). Increased glycogen content is associated with increased phosphorylation, whereas decreased glycogen content decreases GS phosphorylation at several sites (19, 25, 29, 35). Insulin increases GS activity via PI 3-kinase and reduces phosphorylation of GS at sites phosphorylated by GSK-3 (sites 3a, 3b, 3c, and 4) (2, 20, 36).

Muscle contraction activates GS independently of GSK-3 but requires RGL to activate GS (1). Reduced glycogen content after exercise contributes to increased GS activity (33), but muscle contraction additionally increases GS activity independently of glycogen content (29). PP1 activity has been suggested to decrease as glycogen content increases and allow GS phosphorylation (20, 36). Favoring this idea, it has been shown that overexpression of RGL decreases GS phosphorylation and increases glycogen content, whereas the glycogen content is low in muscles where RGL is deleted (1). Glycogen content and muscle contraction are much stronger regulators of GS phosphorylation and activity than insulin (9, 19, 33), which may reflect a more active phosphatase activity and a more comprehensive dephosphorylation of GS than insulin-stimulated inhibition of GSK-3.

Three accompanying papers entitled: “The mechanism of epinephrine action” (6–8) are the experimental background for the Cori cycle stating that “glycogen mobilized in the muscles is converted into liver glycogen with lactic acid as an intermediary stage.” (p. 317 in Ref. 6). The three papers consisted of experiments where epinephrine was injected into rats under different nutritional status, and it was shown that epinephrine directed glycogen to the liver at the expense of carcass glyco-
Importantly, the effect of epinephrine-stimulated glycogen breakdown on GS phosphorylation and activation after removal of epinephrine has not been carefully characterized. The primary aim of the present study was to investigate GS phosphorylation and activation in epitrochlearis muscles following epinephrine-stimulated glycogen breakdown and after removal of epinephrine. The studies were performed in muscles with different glycogen content to investigate whether initial glycogen content influences the magnitude of epinephrine-stimulated glycogen breakdown and GS activation. We hypothesized that skeletal muscle GS phosphorylation and activation would adapt to the new glycogen content via autoregulatory feedback mechanisms after removal of epinephrine.

The glycogen content in skeletal-muscles influences insulin-, contraction-, and pharmacological stimulated glucose uptakes. In rodents, fasting and refeeding (17, 19, 29, 30), acute exercise (10, 11), and training (26) have been used to modulate glycogen content and established an inverse relationship between glycogen content and glucose uptake. In humans, glucose uptake is also regulated by glycogen when glycogen content is modulated by exercise and diet (42). There is also evidence that decreasing glycogen content by epinephrine stimulation increases insulin-stimulated glucose uptake (22, 34). However, these studies do not allow separating the effect of epinephrine-stimulated glycogen breakdown from potential effects independent of glycogen content. The secondary aim was therefore to investigate the role of epinephrine-mediated glycogen breakdown on insulin-stimulated glucose uptake in muscles with different glycogen content.

MATERIALS AND METHODS

Experimental protocol. Male Wistar rats were kept on a 12:12-h light-dark cycles at 21°C and ≈55% humidity with free access to chow and water for at least 1 wk before experiments. Prior to experiments, glycogen content in epitrochlearis muscle was manipulated by fasting and refeeding, as described previously (17). Briefly, rats with low glycogen (LG) were fasted for 24 h prior to experiments. Rats with normal glycogen (NG) were kept on their normal diet until experiments. Rats with high glycogen (HG) were fasted for 24 h followed 24 h with free access to chow prior to experiments (Fig. 1). Fasting was started at 9:00 AM. On experimental days, rats were given a subcutaneous injection of saline (0.9% saline) or epinephrine (0.02 mg/100 g body wt of rats) and placed alone in new cages without food but access to water. Epinephrine [epinephrine (+) bitratrate, E4375; Sigma, St. Louis, MO] was dissolved in 0.9% saline (0.1 mg/ml), and 0.2 ml/100 g body wt was injected subcutaneously into the back of the rats. Muscles used for incubation were removed 3 h after saline/epinephrine injection. Additional muscles and livers were removed and frozen directly in liquid nitrogen from a group of rats without injection and with free access to food and water until experiments (untreated controls) and from groups of rats 1 h after epinephrine/saline injection. Blood glucose was also measured in these rats. The weight of the rats was 120–150 g on the day of experiment. Rats were anesthetized with an intraperitoneal injection of 7.5 mg of pentobarbital sodium (50 mg/ml) per 100 g of rat 3 h after epinephrine/saline injection. Blood glucose was also measured in these rats.

Muscles and liver tissue were stored at −70°C until analyses. The experiments and procedures were approved by National Animal Research Authority and performed according to 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.

Incubations. Epitrochlearis muscles were immediately mounted on holders at their approximate resting length and preincubated ~45 min in 3.5 ml of Krebs-Henseleit buffer containing 5.5 mM glucose, 2 mM pyruvate, 0.1% bovine serum albumin (Fraction V), and 5 mM HEPES, as described (27). The incubations were performed at 30°C, and the buffers were gassed with 95% O₂-5% CO₂.

After preincubation, glucose uptake was measured for 30 min as described previously (27). In brief, 0.25 μCi/ml 2-deoxy-D-[1,2-3H(N)]glucose (25.5 Ci/mmol; DuPont, NEN) and 0.1 μCi/ml D-[1-14C]mannitol (51.5 mCi/mmol; DuPont, NEN) were added to the
buffer (containing 5.5 mM glucose), and glucose uptake was calculated from the intracellular accumulation of \( ^{3}H \)-2-deoxy-D-glucose as described previously (15). Insulin (Actrapid, Novo Nordisk) was added in concentrations of \( 100 \mu \text{U/mL} \) (physiological concentration) or \( 10,000 \mu \text{U/mL} \), as indicated in figures. Muscles were, after the 30-min incubation, rapidly removed from the holders, avoiding unnecessary stretching, blotted on filter paper, and frozen in liquid nitrogen. For measurement of glucose uptake and glycogen, about one-half the muscle was freeze-dried and weighed and dissolved in 600 \( \mu \text{l} \) of 1 M KOH for 20 min at 70°C. Of the digest, 400 \( \mu \text{l} \) was added to 3 ml of scintillation solution (Hionic-Fluor, Packard), mixed, and counted for radioactivity (TRI-CARB 460C, Packard).

Glycogen content in muscles. For analysis of glycogen, 100 \( \mu \text{l} \) of the KOH digest was acidified with 25 \( \mu \text{l} \) of 7 M acetic acid before 500 \( \mu \text{l} \) of 0.3 M acetate buffer (\( \text{pH} 4.8 \)) containing 0.2 \( \mu \text{l} \) of amylglucosidase (Boehringer-Mannheim) was added, and the glycogen was hydrolyzed at 37°C for 3 h. Glucose units were measured enzymatically with reactions (hexokinase and glucose-6-phosphate dehydrogenase) coupled to NADPH formation as described by Lowry and Passonneau (32). Reactions were allowed for 30 min at room temperature in 96-well plates, and fluorescence was measured on a BMG plate reader (BMG, Germany).

Glycogen content in liver. Liver samples were freeze-dried and weighed, and glycogen was hydrolyzed in 1 M HCl (2.5 h at 100°C). Hydrolysat was neutralized with NaOH as described (23).

Homogenization of muscles. Epitrochlearis muscle tissue (~10 mg) was homogenized in buffer containing 50 mM HEPES (\( \text{pH} 7.5 \)), 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 1% NP-40, 20 mM \( \beta \)-glycerol phosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10 \( \mu \text{g/l} \) aprotinin, 10 \( \mu \text{g/l} \) leupeptin, 2 mM Na\(_3\)VO\(_4\), and 3 mM benzamidine. Homogenates were rotated for 1 h at 4°C. Protein content was analysed in triplicates in 96-well plate with the 600

protein content was analysed in triplicates in 96-well plate with the

600

one-half the muscle was freeze-dried and weighed and dissolved in

600

the KOH digest was acidified with 25 \( \mu \text{l} \) of 7 M acetic acid before

600

500

l was

600

l was

600

l of 1 M KOH for 20 min at 70°C. Of the digest, 400 \( \mu \text{l} \) was

600

the KOH digest was acidified with 25 \( \mu \text{l} \) of 7 M acetic acid before

600

the KOH digest was acidified with 25 \( \mu \text{l} \) of 7 M acetic acid before

600

l was

600

l of 1 M KOH for 20 min at 70°C. Of the digest, 400 \( \mu \text{l} \) was

600

the KOH digest was acidified with 25 \( \mu \text{l} \) of 7 M acetic acid before

600

the KOH digest was acidified with 25 \( \mu \text{l} \) of 7 M acetic acid before

600

l was

600

l was

600

l was

600

l was

600

l was

600

l of 1 M KOH for 20 min at 70°C. Of the digest, 400 \( \mu \text{l} \) was

600

The antibodies used to study phosphorylation of GSK-3β, AMPK, and TBC1D4 were described (15). Insulin was added to 3 ml of scintillation solution (Hionic-Fluor, Packard), mixed, and counted for radioactivity (TRI-CARB 460C, Packard).

Glycogen content in muscles. For analysis of glycogen, 100 \( \mu \text{l} \) of the KOH digest was acidified with 25 \( \mu \text{l} \) of 7 M acetic acid before 500 \( \mu \text{l} \) of 0.3 M acetate buffer (\( \text{pH} 4.8 \)) containing 0.2 \( \mu \text{l} \) of amylglucosidase (Boehringer-Mannheim) was added, and the glycogen was hydrolyzed at 37°C for 3 h. Glucose units were measured enzymatically with reactions (hexokinase and glucose-6-phosphate dehydrogenase) coupled to NADPH formation as described by Lowry and Passonneau (32). Reactions were allowed for 30 min at room temperature in 96-well plates, and fluorescence was measured on a BMG plate reader (BMG, Germany).

Homogenization of muscles. Epitrochlearis muscle tissue (~10 mg) was homogenized in buffer containing 50 mM HEPES (\( \text{pH} 7.5 \)), 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 1% NP-40, 20 mM \( \beta \)-glycerol phosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10 \( \mu \text{g/l} \) aprotinin, 10 \( \mu \text{g/l} \) leupeptin, 2 mM Na\(_3\)VO\(_4\), and 3 mM benzamidine. Homogenates were rotated for 1 h at 4°C. Protein content was analysed in triplicates in 96-well plate with the bicinechonic acid method (Pierce Chemical, Rockford, IL).

GS activity. GS activity was measured with the filter paper method and \( \text{[14C]UDPGlucose} \) (PerkinElmer) as substrate as described by Thomas et al. (37), but modified to 96-well filter plates (Unifilter 350 plates; Watman, Cambridge, UK) as described (15). GS activity was measured with 1.5 mM UDP-glucose (high) and 0.03 mM UDP-glucose (physiological) in assay buffer and different concentrations of glucose 6-phosphate. Total GS activity was measured with 8 mM glucose 6-phosphate. GS activity measured with 0.17 mM glucose 6-phosphate was used to calculate fractional velocity \( \text{[FV} = \text{(activity with 0.17 mM G6P)/activity with 8 mM G6P}] \). GS activity measured with 0.01 mM glucose 6-phosphate was used to calculate GS I-form \( \text{[I-form} = \text{(activity with 0.01 mM G6P)/activity with 8 mM G6P}] \).

Western blot. Proteins were separated using 10% SDS gels and transferred (semidyrid) to polyvinylidene difluoride (PVDF) membranes (Immolab Transfer Membrane; Millipore, Copenhagen, Denmark). Membranes were probed with antibodies against GS sites 2+2a, 3a+3b, and 1b as described (15). The antibodies used to study phosphorylation of GSK-3β, AMPK, and TBC1D4 were described (15). Insulin (Actrapid, Novo Nordisk) was added in concentrations of \( 100 \mu \text{U/mL} \) (physiological concentration) or \( 10,000 \mu \text{U/mL} \), as indicated in figures. Muscles were, after the 30-min incubation, rapidly removed from the holders, avoiding unnecessary stretching, blotted on filter paper, and frozen in liquid nitrogen. For measurement of glucose uptake and glycogen, about one-half the muscle was freeze-dried and weighed and dissolved in 600 \( \mu \text{l} \) of 1 M KOH for 20 min at 70°C. Of the digest, 400 \( \mu \text{l} \) was added to 3 ml of scintillation solution (Hionic-Fluor, Packard), mixed, and counted for radioactivity (TRI-CARB 460C, Packard).

Glycogen content in muscles. For analysis of glycogen, 100 \( \mu \text{l} \) of the KOH digest was acidified with 25 \( \mu \text{l} \) of 7 M acetic acid before 500 \( \mu \text{l} \) of 0.3 M acetate buffer (\( \text{pH} 4.8 \)) containing 0.2 \( \mu \text{l} \) of amylglucosidase (Boehringer-Mannheim) was added, and the glycogen was hydrolyzed at 37°C for 3 h. Glucose units were measured enzymatically with reactions (hexokinase and glucose-6-phosphate dehydrogenase) coupled to NADPH formation as described by Lowry and Passonneau (32). Reactions were allowed for 30 min at room temperature in 96-well plates, and fluorescence was measured on a BMG plate reader (BMG, Germany).

Glycogen content in liver. Liver samples were freeze-dried and weighed, and glycogen was hydrolyzed in 1 M HCl (2.5 h at 100°C). Hydrolysate was neutralized with NaOH as described (23).
levels during 24-h fasting, and epinephrine injection increased glycogen content by ~100 mmol/kg dry wt (P < 0.01; Table 1). Fasting-refed increased liver glycogen content to ~ 1,300 mmol/kg dry wt, and epinephrine injection did not influence glycogen content (Table 1).

Glycogen content in epitrochlearis muscles from rats on normal diet was ~150 mmol/kg dry wt 3 h after saline infusion, whereas glycogen content 3 h after epinephrine infusion was decreased to ~100 mmol/kg dry wt (Fig. 3A). In epitrochlearis from fasted-refed rats, the glycogen content was higher than in epitrochlearis from rats kept on normal diet and was ~200 mmol/kg dry wt 3 h after saline infusion. Three hours after epinephrine injection, glycogen content was reduced to ~120 mmol/kg dry wt in epitrochlearis muscles from fasted-refed rats, and glycogen content tended to be higher than in muscles from rats on a normal diet injected with epinephrine (P < 0.1). In epitrochlearis muscles from fasted rats glycogen contents was ~100 mmol/kg dry wt, and epinephrine injection did not decrease glycogen content significantly (Fig. 3A).

GS activity was influenced by glycogen content in saline-injected rats, when measured with both physiological and high concentration of UDP-glucose (Fig. 3, B–E). GS fractional activities and GS I-form percent measured with 0.03 mM UDP-glucose (FV0.03 and I-form0.03) were, as expected, much higher than in epitrochlearis from rats kept on normal diet and

![Graphs showing effect of epinephrine injection on glycogen content, glycogen synthase (GS) activation, and phosphorylation of GS, GSK-3β, and AMPK in epitrochlearis muscles from rats under different nutritional conditions.](http://ajpendo.physiology.org/)

Fig. 3. Effect of epinephrine injection on glycogen content, glycogen synthase (GS) activation, and phosphorylation of GS, GSK-3β, and AMPK in epitrochlearis muscles from rats under different nutritional conditions. Rats were diet manipulated as described in MATERIALS AND METHODS. Saline or epinephrine (0.02 mg/100 g rat) was injected subcutaneously, and epitrochlearis muscles were removed 3 h later and preincubated for 45 min and incubated for 30 min in control buffer.
lower than activities measured with 1.5 mM UDP-glucose, but the effect of glycogen content was comparable. GS activity was increased in muscles from epinephrine-injected rats on normal diet and fasted-refed (Fig. 3, B–E). In epitrochlearis muscles from fasted rats, GS activity was not increased in muscles from epinephrine-injected rats; of note, epinephrine did not decrease glycogen content in these muscles. Some epitrochlearis muscles were removed 1 h after epinephrine injection and frozen immediately to describe the acute effect of epinephrine injection; epinephrine injection decreased GS activity as expected in all groups, although not significantly in epitrochlearis from fasted-refed rats (Table 2).

Phosphorylation of GS sites 2+2a and 3a+3b were influenced by modulation of glycogen content by fasting and fasting-refeeding (Fig. 3, F and G). GS phosphorylation at sites 2+2a and 3a+3b was reduced in epitrochlearis muscles from epinephrine-injected rats on normal diet and fasted-refed (Fig. 3, F and G); epinephrine injection decreased glycogen content in muscles from these groups of rats (Fig. 3A). GS site 1b phosphorylation was higher in epitrochlearis from fasted-refed rats compared with muscles from fasted rats (Fig. 3H). GS site 1b phosphorylation was not regulated by epinephrine injection (Fig. 3H).

GSK-3β Ser9 phosphorylation was higher in epitrochlearis from fasted-refed rats on normal diet and fasted-refed (Fig. 3A). AMPK Thr172 phosphorylation was increased in epitrochlearis from fasted-refed rats compared with both rats on normal diet and from fasted-refed rats (Fig. 3I). AMPK Thr172 phosphorylation was similar in muscles from saline- and epinephrine-injected rats (Fig. 3J).

Insulin increased GS activation in epitrochlearis from saline-injected rats (Fig. 4). However, insulin-stimulated GS activation was observed mainly when GS activities were measured with physiological concentration of UDP-glucose. GS FV 0.03 and GS I-form 0.03 were increased by insulin in epitrochlearis from rats on normal diet and from fasted rats injected with saline (Fig. 4, G, H, J, and K). Insulin only increased GS GS I-form 1.5 in epitrochlearis muscles from rats on normal diet (Fig. 4B), and insulin did not increase FV 1.5 significantly in any groups (Fig. 4, D–F). In epitrochlearis muscles from epinephrine-injected rats, insulin increased GS activation in all groups independently of glycogen content when measured with 0.03 mM UDP-glucose (Fig. 4, G–L). With a high concentration of UDP-glucose, insulin increased GS I-form percent in epitrochlearis from epinephrine-injected rats on normal diet (Fig. 4B).

Insulin did not influence GS site 2+2a phosphorylation in any of the groups (Fig. 5, A–C). Insulin decreased GS site 3a+3b phosphorylation in epitrochlearis muscles with low and normal glycogen content but not in epitrochlearis from fasted-refed rats injected with saline (Fig. 5, D–F). GS site 3a+3b phosphorylation was lower in epitrochlearis from epinephrine-injected rats in all groups also during insulin stimulation except in muscles from fasted rats in the presence of 10,000 μU/ml insulin (5D). In epitrochlearis muscles from epinephrine-injected rats on normal diet or fasted rats, insulin stimulation tended to decrease GS site 3a+3b phosphorylation (P < 0.1; Fig. 5, D and E). Insulin did not decrease GS site 3a+3b phosphorylation significantly in epinephrine-injected epitrochlearis muscles from fasted-refed rats (Fig. 5F). GS site 1b phosphorylation was not regulated by insulin (Fig. 5, G–I).

Insulin increased GSK-3β Ser9 phosphorylation similarly in epitrochlearis muscles from fasted, fasted-refed, and normal-diet rats independently of epinephrine injection (Fig. 5, J–L). Incubation with insulin did not influence AMPK Thr172 phosphorylation (data not shown).

Basal and insulin-stimulated glucose uptake was higher in epitrochlearis muscles from fasted rats than in muscles from rats on normal diet and fasted-refed rats when saline was injected. Insulin increased glucose uptake in all groups (Fig. 6, B, E, and H). Insulin-stimulated glucose uptake was higher in epitrochlearis muscles from epinephrine-injected rats on normal diet and fasted-refed rats (Fig. 6, E and H); epinephrine injection decreased glycogen content in these muscles (Fig. 6, D and G). In muscles from fasted rats, glucose uptake was similar in saline- and epinephrine-injected rats when measured with 0 (basal) and 10,000 μU/ml of insulin (Fig. 6B). Glucose uptake was higher in epitrochlearis muscles from epinephrine-injected measured with 100 μU/ml of insulin (Fig. 6B). Of note, glycogen content was lower in epitrochlearis muscles from fasted rats after epinephrine injection at this insulin concentration (Fig. 6A). Insulin increased TBC1D4 Thr422 phosphorylation in muscles independently of glycogen content (Fig. 6, C, F, and I). Furthermore, insulin-stimulated TBC1D4 Thr422 phosphorylation was similar in muscles from saline- and epinephrine-injected rats despite the glucose uptake being higher when glycogen content was reduced by epinephrine injection.

**DISCUSSION**

It is well documented that glycogen content influences GS phosphorylation and activity, but the mechanisms linking glycogen content to regulation of GS activity remain elusive. Most previous studies have manipulated glycogen content by a combination of exercise and diet or fasting-refeeding protocols. Therefore, to further understand the role of glycogen content on GS phosphorylation and activation, epinephrine-stimulated glycogen breakdown was initiated in muscles with different glycogen content to test the hypothesis that GS phosphorylation adapts to reduced glycogen content when epinephrine is removed. GS phosphorylation was decreased and GS activation increased in epitrochlearis muscles with epinephrine-stimulated glycogen breakdown. Importantly, epinephrine did not decrease glycogen content in muscles with low glycogen content, and no effect on GS phosphorylation or activation was observed in these muscles, showing a condition during which epinephrine stimulation per se does not modulate GS phosphorylation and activation.

The primary aim of the present study was to investigate the effect of epinephrine-mediated glycogen breakdown on GS phosphorylation and GS activity. Epinephrine activates PKA,
and the kinase phosphorylates GS at sites 2a, 1a, and 1b (5, 18, 36, 38). PKA also phosphorylates the PP1 binding protein RGL, which releases PP1 from the glycogen particle and increases GS activity. GS phosphorylation is regulated independent of GSK-3β (19) have previously shown that when glycogen content is manipulated by fasting-refeding GS phosphorylation is regulated independently of GSK-3β. Indeed, reduction in glycogen content by fasting requires epinephrine (41), and our data show that both fasting and epinephrine injection decreased GS phosphorylation 

![Figure 4](http://ajpendo.physiology.org/)

**Fig. 4.** Effects of epinephrine injection on insulin-stimulated GS activation in epitrochlearis muscles from rats under different nutritional conditions. Rat were diet manipulated as described, saline or epinephrine (0.02 mg/100 g rat) was injected subcutaneously, and epitrochlearis muscles were removed 3 h later and preincubated for 45 min and incubated 30 min in buffer without and with different concentrations of insulin. A–C: GS 1-Form 0.03 in epitrochlearis muscles from 24-h-fasted rats (F), rats on normal diet (N), and fasted-refed rats (L) after injection of saline (open bars) or epinephrine (filled bars) and incubated with different concentrations of insulin (0, 100, 10,000 µU/ml). D–F: GS V1-Form 1.5 in epitrochlearis muscles from 24-h-fasted rats (D), rats on normal diet (E), and fasted-refed rats (F) after injection of saline (open bars) or epinephrine (filled bars) and incubated with different concentrations of insulin (0, 100, 10,000 µU/ml). G–I: GS 1-Form 0.03 in epitrochlearis muscles from 24-h-fasted rats (G), rats on normal diet (H), and fasted-refed rats (I) after injection of saline (open bars) or epinephrine (filled bars) and incubated with different concentrations of insulin (0, 100, 10,000 µU/ml). J–L: GS V1-Form 0.03 in epitrochlearis muscles from 24-h-fasted rats (J), rats on normal diet (K), and fasted-refed rats (L) after injection of saline (open bars) or epinephrine (filled bars) and incubated with different concentrations of insulin (0, 100, 10,000 µU/ml). Data are means ± SE; n = 7–8 in all groups. *P < 0.05 vs. muscles from saline-treated rats; †P < 0.05 vs. muscles from fasted rats treated similarly; ‡P < 0.05 vs. normal diet with similar treatment.

Reduced glycogen content. GSK-3β phosphorylates GS sites 3a, 3b, 3c, and 4 (36), but GSK-3β phosphorylation was not regulated by epinephrine injection or by glycogen content. We (19) have previously shown that when glycogen content is manipulated by fasting-refeding GS phosphorylation is regulated independent of GSK-3β. Indeed, reduction in glycogen content by fasting requires epinephrine (41), and our data show that both fasting and epinephrine injection decreased GS phosphorylation independently of GSK-3. Insulin, on the other hand, requires GSK-3 phosphorylation for GS activation (2). In the present study, insulin increased GSK-3β Ser9 phosphorylation.
ylation in muscles from epinephrine-injected rats occurred.

phosphorylation was similar in muscles from saline- and epinephrine, as expected (9, 19, 29, 33).

in muscles where glycogen content was reduced by epinephrine-mediated increase in GS activity was also less than the increase

2a, agreeing with previous

/n

SE; 0.05; (c)

lation and decreased GS site 3a+3b phosphorylation, but not phosphorylation of GS site 2+2a, agreeing with previous studies in rat skeletal muscles (13, 16, 19, 29–31). The insulin-mediated increase in GS activity was also less than the increase in muscles where glycogen content was reduced by epinephrine, as expected (9, 19, 29, 33).

AMPK phosphorylates GS site 2 (25, 36). However, AMPK phosphorylation was similar in muscles from saline- and epinephrine-injected rats, and the lower GS site 2+2a phosphorylation in muscles from epinephrine-injected rats occurred

without regulation of AMPK phosphorylation. Muscle contraction decreases GS phosphorylation at sites 2+2a and 3a+3b (29, 30, 35), as we observed in muscles where epinephrine injection decreased glycogen content. Muscle contraction requires RGL for activation of GS, suggesting that contraction regulates GS phosphorylation via PP1 activation (1). This agrees with the more general dephosphorylation of GS during muscle contraction compared with insulin stimulation (30).

Muscles with high glycogen have reduced protein phosphatase activity, whereas overexpression of RGL increases glycogen

Fig. 5. Effects of epinephrine injection on insulin-stimulated phosphorylation of GS and GSK-3β in epitrochlearis muscles from rats under different nutritional conditions. Rats were diet manipulated as described, saline or epinephrine (0.02 mg/100 g rat) was injected subcutaneously, and epitrochlearis muscles were removed 3 h later and preincubated for 45 min and incubated 30 min in buffer without and with different concentrations of insulin. A–C: GS site 2+2a phosphorylation in epitrochlearis muscles from 24-h-fasted rats (A), rats on normal diet (B), and fasted-refed rats (C) after injection of saline (open bars) or epinephrine (filled bars) and incubated with different concentrations of insulin (0, 100, 10,000 µU/ml). D–F: GS site 3a+3b phosphorylation in epitrochlearis muscles from 24-h-fasted rats (D), rats on normal diet (E), and fasted-refed rats (F) after injection of saline (open bars) or epinephrine (filled bars) and incubated with different concentrations of insulin (0, 100, 10,000 µU/ml). G–I: GS site 1b phosphorylation in epitrochlearis muscles from 24-h-fasted rats (G), rats on normal diet (H), and fasted-refed rats (I) after injection of saline (open bars) or epinephrine (filled bars) and incubated with different concentrations of insulin (0, 100, 10,000 µU/ml). J–L: GSK-3β Ser21 phosphorylation in epitrochlearis muscles from 24-h-fasted rats (J), rats on normal diet (K), and fasted-refed rats (L) after injection of saline (open bars) or epinephrine (filled bars) and incubated with different concentrations of insulin (0, 100, 10,000 µU/ml). Data are means ± SE; n = 7–8 in all groups. *P < 0.05; **P < 0.1 vs. muscles from saline-treated rats; ***P < 0.05; ****P < 0.1 vs. muscles from fasted rats treated similarly; #P < 0.05; $P < 0.1 vs. normal diet with similar treatment.

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00282.2014 • www.ajpendo.org
content (1), supporting the concept that glycogen content regulates GS phosphorylation via PP1. Indeed, epinephrine-stimulated glycogen breakdown did not decrease GS site 1b phosphorylation, but overall, our data support that epinephrine-stimulated glycogen content decreases GS phosphorylation via activation of PP1. We suggest that $R_\text{Gly}$ becomes dephosphorylated after removal of epinephrine and recruits PP1 back to the glycogen particle and dephosphorylates GS in relation to the glycogen content.

This is the first documentation that epinephrine-mediated glycogen breakdown is directly linked to regulation of GS phosphorylation and activation. Indeed, Danforth (9) used epinephrine in one of his experiments to modulate glycogen content to study the relationship between glycogen content and GS activation, but it is not possible to separate effects independent of reduced glycogen content in that study. The data in the present study enable us to separate the effect of glycogen content from that of epinephrine stimulation per se, as epinephrine did not significantly reduce glycogen content in muscles from fasted rats. In muscles from fasted rats, epinephrine did not influence GS phosphorylation and GS activation. Therefore, the present study for the first time documents a direct “autoregulation” of GS phosphorylation by glycogen content.

The modulation of glycogen content in epitrochlearis muscles prior to epinephrine injection also showed that the glycogen content regulates epinephrine-stimulated glycogenolysis. In agreement with a previous study (34), epinephrine injection decreased muscle glycogen content by 45% in muscle from rats on normal diet. Epinephrine did not significantly decrease glycogen content in epitrochlearis from fasted rats, suggesting that muscles with low glycogen content are protected from complete depletion by the stress hormone. Previously, glycogen phosphorylase activation was reported to be lower in glycogen-depleted muscles and increases as glycogen stores are repleted (3, 12). Glycogen phosphorylase activation and glycogenolytic rate are also elevated during muscle contraction
in muscles with high glycogen (14). Although we did not find a significant reduction in glycogen content in epitrochlearis from fasted rats (~10% lower), blood glucose increased after epinephrine injection, and liver glycogen increased, as reported by Cory and Cory (6), showing that epinephrine relocates carbohydrates between organs.

Insulin-stimulated glucose uptake was increased in muscles where epinephrine injection reduced glycogen content, agreeing with previous studies in which glycogen content was modulated by fasting-refeeding or exercise and diet (10, 17, 19). An increase in insulin-stimulated glucose uptake after epinephrine injection has been reported previously (22, 34), but this is the first comprehensive investigation where epinephrine was injected in rats with different glycogen content. In epitrochlearis from rats on normal diet and from fasted-refed rats, where epinephrine reduced glycogen content, glucose uptake was increased at both physiological and high insulin concentration, indicating that insulin sensitivity and responsiveness were both increased. Of note, glucose uptake at a physiological insulin concentration was increased in muscles from fasted rats, but these muscles also had a small reduction in glycogen content, and insulin-stimulated glucose uptake was increased in muscles only where epinephrine stimulated glycogen breakdown. Insulin increased TBC1D4 Thr642 phosphorylation as expected (4, 39). Importantly, insulin-stimulated TBC1D4 Thr642 phosphorylation was similar in muscles from saline- and epinephrine-injected rats despite the fact that insulin-stimulated glycogen was elevated in muscles where epinephrine decreased glycogen content. Therefore, epinephrine-stimulated glycogen content did not influence TBC1D4 Thr642 and GSK-3β Ser9 phosphorylation, indicating that insulin signaling was not amplified at key molecules regulating glucose uptake and GS activity, respectively. The present data therefore support a mechanistic link between glycogen content and stimulation of glucose uptake, although such a mechanism remains to be documented.

In conclusion, epinephrine stimulated glycogen breakdown in epitrochlearis muscles with normal and high glycogen content but not in muscles with low glycogen content. GS phosphorylation decreased and GS activation increased in muscles where epinephrine injection decreased glycogen content. Importantly, epinephrine did not decrease glycogen content in muscles from fasted rats, and GS was neither dephosphorylated nor activated in these muscles. Therefore, our data document for the first time a direct autoregulation of GS phosphorylation by glycogen content after removal of epinephrine. Our data also show that epinephrine-stimulated glycogen breakdown increases insulin-stimulated glucose uptake and therefore that the Cory cycle contributes to increased insulin sensitivity in skeletal muscles.

ACKNOWLEDGMENT

We thank Betina Bolingren, Astrid Bolling, Ada Ingvaldsen, Fang-Chin Lin, David Håkensen, and Kristoffer Cumming for technical assistance. Prof. Erik A. Richter is thanked for most useful comments to the manuscript. This work was carried out as a part of the program of the UNIK: Food, Fitness & Pharma for Health and Disease (see www.foodfitnesspharma.ku.dk).

REFERENCES

28. Lai YC, Lin FC, Jensen J.


