Mechanism of hypoxia-stimulated glucose transport in rat skeletal muscle: potential role of glycogen

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Reynolds, Thomas H., IV, Joseph T. Brozinick, Jr., M. A. Rogers, and Samuel W. Cushman. Mechanism of hypoxia-stimulated glucose transport in rat skeletal muscle: potential role of glycogen. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E773–E778, 1998.—We have previously reported that exercise training is associated with enhanced insulin-stimulated glucose transport activity and inhibited hypoxia-stimulated glucose transport activity in rat epitrochlearis muscle. Here we examine the potential role of muscle glycogen in the inhibited glucose transport response to hypoxia. Three days of swim training (2 × 3 h/day) produce a 100% increase in glycogen and a 70% increase in GLUT-4 in epitrochlearis muscle. Glucose transport after 1 h of hypoxia in muscles from fed exercise-trained (ET) rats is not significantly elevated above basal and is 40% lower than that in muscles from fed sedentary (SED) rats. Glycogen levels after 1 h of hypoxia are reduced by 27 and 64% in muscles from fed ET and fed SED rats, respectively. After 2 h of hypoxia, glucose transport is significantly increased above basal in muscles from fed ET rats, but this response is still 55% lower than that in muscles from fed SED rats. After 2 h of hypoxia, glycogen is reduced by 50 and 83% in muscles from fed ET and fed SED rats, respectively. After a modified overnight fast (=4.5 g of chow), the glucose transport and glycogen responses to 1 h of hypoxia are not significantly different between muscles from ET and SED rats. These findings demonstrate a strong inverse relationship between glycogen and hypoxia-stimulated glucose transport activity and that high levels of glycogen contribute to the inhibited glucose transport response to hypoxia. Furthermore, failure of the overexpression of GLUT-4 after exercise training to enhance the glucose transport response to hypoxia suggests selective targeting of the additional GLUT-4 to the insulin-responsive pool.

Glucose transport across the plasma membrane is thought to be the rate-limiting step in glucose metabolism and appears to be mediated by the translocation of intracellular GLUT-4 to the cell surface, as is now well established in adipose cells (8, 34). In skeletal muscle, insulin (21, 23, 36) and contraction/hypoxia (4, 22) appear to induce the translocation of intracellular GLUT-4 to the plasma membrane, at least as evidenced by the appearance of GLUT-4 on the cell surface. Several laboratories have reported that the glucose transport response to the combined effects of insulin and contraction/hypoxia is additive (4, 26, 35) and thus have proposed that skeletal muscle contains two pools of GLUT-4, one responsive to insulin and the other responsive to contraction/hypoxia (1, 6, 10). However, these same laboratories have also reported the contrary (7, 11, 28). The precise experimental conditions used in these studies are likely to be a critical factor in explaining this disparity.

Endurance exercise training has consistently been shown to increase skeletal muscle glycogen levels, total muscle GLUT-4 content, and insulin-stimulated glucose transport activity (29, 30, 32). However, the effects of exercise training on the glucose transport response to contraction/hypoxia have been inconsistent. Ren et al. (30) demonstrated that exercise training increases both contraction- and hypoxia-stimulated glucose transport in white muscle. In contrast, other investigations have reported that exercise training does not alter contraction-stimulated glucose transport activity in white muscle (2, 29). Recently, we have shown that exercise training inhibits hypoxia-stimulated glucose transport activity and the appearance of cell surface GLUT-4 in isolated epitrochlearis muscle, despite a significant elevation in total muscle GLUT-4 content (31).

In the present study, we have manipulated muscle glycogen levels by exercise training and food restriction, and we have examined the glucose transport and glycogen responses to hypoxia. Our results demonstrate that muscle glycogen and GLUT-4 levels do not regulate basal glucose transport. However, when muscle glycogen levels are elevated by exercise training, decreasing glycogen levels in response to hypoxia progressively stimulate glucose transport and establish a strong inverse relationship between glycogen levels and glucose transport activity. Finally, our results indicate that the contraction/hypoxia-responsive pool of GLUT-4 and corresponding glucose transport response to contraction/hypoxia are not enhanced by exercise training.
METHODS

Animals and housing. Specific pathogen-free male Wistar rats weighing 55 g were obtained from Charles River Laboratories (Wilmington, MA). On arrival, rats were housed four per cage in a temperature-controlled animal room maintained on a 12:12-h light-dark cycle. The rats were fed ad libitum National Institutes of Health (NIH) standard chow and water. Two days after arrival, the rats were randomly assigned to an exercise training (ET) or sedentary control (SED) group.

ET protocol. The rats assigned to the ET group were acclimated to swimming for 10 min/day for 2 days and then exercise-trained by a procedure similar to that used by Ploug et al. (29) and Ren et al. (30). Briefly, the animals performed two bouts of exercise, separated by a 1-h rest period, in water maintained at 35°C. During the rest period, animals were towel dried, kept warm, and given food and water. The rats swam in groups of six in 130-liter plastic barrels (50 × 70 cm, diameter × height). On the 1st day of training, the rats performed 1-h and 2-h bouts of swimming. On the 2nd day of training, the swimming duration was increased so that the animals performed two 3-h bouts of exercise. The latter regimen was repeated for a total of 3 days. This protocol was approved by the NIH Animal Care and Use Committee.

Muscle preparation and incubation. At 18–22 h after the last bout of exercise, rats in the postprandial state were anesthetized with pentobarbital sodium (5 mg/100 g body wt). For experiments that involved food restriction, the ET animals were fasted between exercise bouts on the last day of training, and food intake was limited to 4.5 g overnight (semi-fast). The epitrochlearis muscles were dissected out, blotted on gauze, and transferred to 25-ml Erlenmeyer flasks (1 muscle/flask) containing 2 ml of Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 32 mM mannitol, and 8 mM glucose with or without 13.3 mM (2,000 µU/ml) insulin. The flasks were incubated for 1 h at 35°C in a shaking water bath and were continually gassed with 95% N2-5% CO2. After the initial incubation, muscles were blotted and transferred to 25-ml Erlenmeyer flasks (1 muscle/flask) containing 2 ml of Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 32 mM mannitol, and 8 mM glucose with or without 13.3 mM (2,000 µU/ml) insulin. The flasks were incubated for 10 min at 29°C in a shaking water bath to wash out the glucose; the gas phase in all of the flasks was 95% O2-5% CO2.

Measurement of glucose transport activity. The measurement of glucose transport activity has previously been described (5). After the above incubations, muscles were frozen between tongs cooled to the temperature of liquid nitrogen and stored at −70°C until processing. Muscles were homogenized in 0.3 M perchloric acid on ice in Kontes (Vineland, NJ) glass-on-glass grinding tubes, and glycogen levels were determined by the amyloglucosidase method described by Passonneau and Lauderdale (25).

Measurement of total muscle GLUT-4. Freeze-clamped epitrochlearis muscles were weighed and homogenized at 1:40 (wt/vol) in HEPES-EDTA-sucrose buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, 25 mM Benzmamidine, 1 µM leupeptin, 1 µM aprotinin, 1 mM pepstatin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, pH 7.4) on ice. The protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL) with crystalline BSA as the standard. An aliquot of homogenate containing 40 µg of protein was solubilized in Laemml sample buffer and subjected to 10% SDS-PAGE. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes and incubated overnight at 4°C with a rabbit polyclonal anti-GLUT-4 antiserum (kindly prepared by Hoffmann-La Roche, Nutley, NJ). The PVDF membranes were then incubated in 0.25 µCi/ml of 125I-labeled protein A (New England Nuclear) for 1 h at 37°C and placed in a Phosphorimager cassette (Molecular Dynamics, Sunnyvale, CA) for 72 h. After correction for background, the areas of each band were expressed as a percentage of a standard (40 µg of rat skeletal muscle intracellular membrane protein) run on each gel.

Measurement of citrate synthase activity. Aliquots of the same epitrochlearis muscle homogenates used for the measurement of GLUT-4 were used for the determination of citrate synthase activity as described by Serre (33).

Statistical analysis. Statistically significant differences in glucose transport activity and glycogen levels between ET and SED groups were detected by factorial analysis of variance (ANOVA). Because of heterogeneity of variances, glyco-
gen values for muscles from ET and SED animals after a modified overnight fast were rank transformed before the analysis. After a significant F ratio, selected mean comparisons were performed to locate significant differences. A one-way ANOVA was utilized to detect statistically significant differences between ET and SED groups for citrate synthase activity and total GLUT-4 levels. Results are expressed as means ± SE, and differences are accepted as significant at the P ≤ 0.05 level.

RESULTS

Animal body weights and epitrochlearis muscle weights. The ET animals weighed significantly less than the SED animals (101.8 ± 1.0 vs. 114.9 ± 1.4 g), and the epitrochlearis muscles from ET animals weighed significantly less than muscles from SED animals (18.08 ± 0.28 vs. 19.88 ± 0.33 mg).

Citrate synthase activity and total GLUT-4 content. Table 1 illustrates that 3 days of exercise training produced a significant 35% increase in citrate synthase activity and a 70% increase in total muscle GLUT-4 content.

Glucose transport activity. The insulin-stimulated glucose transport activity values are shown in Table 2; the hypoxia-stimulated glucose transport activity val-
ues are illustrated in Fig. 1A. Basal glucose transport activity in epitrochlearis muscle was not altered by 3 days of exercise training or by a modified overnight fast. Insulin-stimulated glucose transport activity was 60 and 50% higher in muscles from ET animals vs. SED animals in the fed and semi-fasted states, respectively. However, the modified overnight fast alone had no significant effect on the glucose transport response to insulin in muscles from either ET or SED animals.

In the fed state, the glucose transport response to 1 h of hypoxia was significantly increased above basal levels in muscles from SED but not ET animals. In the semi-fasted state, the glucose transport responses to 1 h of hypoxia were the same in muscles from ET and SED animals and were identical to those of muscles from fed SED animals exposed to 2 h of hypoxia. Furthermore, 2 h of hypoxia produced a significant increase in glucose transport activity above basal in muscles from fed ET animals, but this response was still 55% lower than that observed in muscles from fed SED animals. Additionally, 2 h of hypoxia produced significant increases in glucose transport activity above 1 h of hypoxia in muscles from both ET and SED fed animals.

Muscle glycogen levels. The basal and hypoxia-stimulated glycogen values are illustrated in Fig. 1B. Basal glycogen levels were twofold higher in epitrochlearis muscle from ET animals compared to fed SED animals. Muscle glycogen levels after 1 h of hypoxia declined by 27 and 65% in muscles from ET and SED animals, respectively. Therefore, after 1 h of hypoxia, muscle glycogen levels were 4.5-fold higher in muscles from ET animals compared to those from SED animals. Furthermore, 2 h of hypoxia produced further 23 and 19% declines in glycogen levels in muscles from fed ET and SED animals, respectively. Again, after 2 h of hypoxia, muscle glycogen levels were 6.5-fold higher in muscles from ET rats compared with those from SED rats. In contrast, basal and hypoxia-stimulated glycogen levels were not significantly different in muscles from ET and SED animals after a modified overnight fast (Fig. 1). This modified overnight fast depleted basal muscle glycogen levels markedly compared with the fed state in muscles from ET rats but hardly at all in muscles from SED animals.

**DISCUSSION**

The present study demonstrates that exercise training produces a differential effect on insulin- and hypoxia-stimulated glucose transport activity. Three days of swim training induce a 50–60% enhancement in the glucose transport response to maximal concentrations of insulin but almost completely obliterate the glucose transport response to hypoxia. The increase in insulin's action after exercise training appears to be indepen-

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**Table 1.** Citrate synthase activity and total GLUT-4 content of epitrochlearis muscles from exercise-trained and sedentary control animals

<table>
<thead>
<tr>
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<th>Control</th>
<th>Exercise Trained</th>
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<tr>
<td>Citrate synthase, µmol·min⁻¹·g⁻¹</td>
<td>21.82 ± 0.02 (12)</td>
<td>29.52 ± 0.93* (11)</td>
</tr>
<tr>
<td>GLUT-4, %standard</td>
<td>18.00 ± 0.01 (10)</td>
<td>30.70 ± 0.80* (10)</td>
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Values are means ± SE of nos. of muscles indicated within parentheses. *Significantly different from control muscles.

**Table 2.** Basal and insulin-stimulated glucose transport activity in epitrochlearis muscles from exercise-trained and sedentary control animals

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Control</th>
<th>Exercise Trained</th>
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<tr>
<td>Fed basal</td>
<td>0.15 ± 0.02 (10)</td>
<td>0.23 ± 0.02 (4)</td>
</tr>
<tr>
<td>Fasted basal</td>
<td>0.26 ± 0.04 (10)</td>
<td>0.39 ± 0.05 (7)</td>
</tr>
<tr>
<td>Fed insulin</td>
<td>0.97 ± 0.11* (8)</td>
<td>1.55 ± 0.09† (8)</td>
</tr>
<tr>
<td>Fasted insulin</td>
<td>1.20 ± 0.33* (8)</td>
<td>1.82 ± 0.09* (8)</td>
</tr>
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Glucose transport values are means ± SE for nos. of muscles indicated within parentheses expressed in µmol·ml⁻¹·20 min⁻¹. *Significantly different from basal muscles. †Significantly different from control muscles.
sistent with muscle glycogen levels, although a slight but not significant increase in the glucose transport response to insulin is observed in muscles from animals that were food restricted overnight. In contrast, the inhibition of hypoxia-stimulated glucose transport activity after exercise training is associated with elevated muscle glycogen levels. Furthermore, exercise training and a modified overnight fast do not alter basal glucose transport, indicating that nonstimulated glucose transport is independent of muscle glycogen levels.

Exercise training has consistently been shown to increase total muscle GLUT-4 levels in both human subjects (18) and rats (30, 32). Several studies have also documented a high correlation between total muscle GLUT-4 levels and maximal insulin- and/or contraction-stimulated glucose transport capacity (14, 20, 32). Although the exact elevation in GLUT-4 levels after exercise training can vary (25–100%) from study to study, the present study demonstrates a 70% increase in total muscle GLUT-4 levels and a 50–60% increase in insulin-stimulated glucose transport activity after just 3 days of exercise training. The adaptive response in GLUT-4 to exercise training was originally thought to take several weeks to occur. However, more recently, skeletal muscle GLUT-4 levels have been shown to be elevated after short-term exercise training (12, 30, 31). Because high-intensity, acute exercise induces considerable muscle glycogen breakdown, the rapid increase in GLUT-4 allows for greater insulin-stimulated glucose transport and subsequent glycogen synthesis (30).

In contrast to the enhancement in the glucose transport response to insulin, the results of the present study show an exercise training-related inhibition of hypoxia-stimulated glucose transport activity despite the large increase in total muscle GLUT-4 levels. Previous investigations have reported that exercise training either does not increase contraction-stimulated glucose transport (2, 29) or actually inhibits hypoxia-stimulated glucose transport and cell surface GLUT-4 in white, glycolytic fibers (31). Because the elevation of muscle glycogen is a hallmark adaptation to exercise training, it is possible that high levels of glycogen can regulate the glucose transport response to contraction/hypoxia (15, 31). The idea that glycogen regulates the glucose transport response to contraction/hypoxia is supported by evidence demonstrating that carbohydrate restriction or fasting prolongs the increase in basal glucose transport after exercise (5, 37). In contrast, carbohydrate feeding speeds the reversal of the enhanced basal glucose transport after exercise (37).

To further explore the role that muscle glycogen plays in regulating hypoxia-stimulated glucose transport, the present study examined the glucose transport and muscle glycogen responses to 2 h of hypoxia. Glucose transport activity is elevated above basal levels in muscle from ET animals with 2 h of hypoxia, thus at least partially reversing the inhibition observed after 1 h of hypoxia. Likewise, 2 h of hypoxia produce a twofold greater glycogen depletion than 1 h of hypoxia. The present study also reports that an overnight modified fast not only equalizes basal glycogen levels between muscles from ET and SED animals but also restores the glucose transport and muscle glycogen responses to hypoxia in muscles from ET animals. Taken together, these findings suggest that a threshold of glycogen depletion (i.e., 50–60% of resting glycogen levels) is necessary for a significant glucose transport response to hypoxia and that a strong inverse relationship exists between glycogen and hypoxia-stimulated glucose transport thereafter (Fig. 2). Because glycogenolytic has been shown to be accelerated when muscle glycogen levels are elevated (16), the present inhibition in the glucose transport response to hypoxia in muscle from ET animals might be due to an accumulation of glycogen 6-phosphate (G-6-P) and an inhibition of hexokinase activity (15). However, although this possibility cannot be ruled out by the current results, it appears unlikely, because the relative degree (i.e., % of resting glycogen) of glycogen breakdown is reduced with exercise training in response to hypoxia. In addition, Jensen et al. (19) demonstrated that, across a wide range of glycogen values, G-6-P-mediated hexokinase inhibition does not alter glucose transport activity. An alternative explanation is that muscle glycogen particles can interact with GLUT-4 vesicles that are responsive to contraction/hypoxia and that glycogen depletion might be essential for contraction/hypoxia to stimulate glucose transport activity, but no experimental evidence is yet available regarding this possibility.

Interestingly, although a modified overnight fast normalizes the response to hypoxia in muscles from ET rats, the glucose transport response to hypoxia in muscles from ET animals does not exceed that in muscles from SED animals despite 70% more GLUT-4. These findings suggest that factors in addition to muscle glycogen regulate the glucose transport re-
sponse to hypoxia in exercise-trained muscle. It is possible that exercise training produces an increased expression of GLUT-4 specifically in the insulin-responsive pool and not in the contraction/hypoxia-responsive GLUT-4 pool. This concept is supported by previous studies demonstrating that exercise training has no effect on contraction-stimulated glucose transport despite an increase in insulin action and total muscle GLUT-4 (2, 29). Additionally, Brozinick et al. (3) have shown that transgenic mice overexpressing GLUT-4 in skeletal muscle exhibit enhanced insulin- but not contraction-stimulated glucose transport.

In muscle from SED animals, hypoxia also produces a significant increase in glucose transport activity and extensive glycogen depletion. However, because of the narrow range of glycogen values in muscle from SED animals, we were unable to establish a correlative relationship between hypoxia-stimulated glucose transport activity and glycogen. Furthermore, basal and insulin-stimulated glucose transport activity appears to be far less dependent on glycogen. In fact, we demonstrate that a modified overnight fast produces a dramatic decrease in basal glycogen but that it only slightly and not significantly increases basal (Fig. 2) and insulin-stimulated glucose transport in muscles from ET animals. In contrast to our results, Jensen et al. (19) reported that glycogen levels are inversely related to basal and insulin-stimulated glucose transport activity. Other studies have also reported that insulin-stimulated glucose transport was higher in muscles with low levels of glycogen (9, 38). Alternatively, Nolte et al. (24) demonstrated that epinephrine-induced muscle glycogen depletion does not enhance the glucose transport response to maximal concentrations of insulin. The present finding that glycogen depletion does not significantly alter basal and insulin-stimulated glucose transport activity might be due to the fasting regimen used to lower muscle glycogen. We used a modified overnight fast (4.5 g of chow) to lower muscle glycogen, whereas previous studies used prolonged fasting (19) or fat feeding (37). Interestingly, the present fasting regimen did not lower glycogen levels in muscles from SED animals; therefore, our findings that a modified overnight fast does not alter basal and insulin-stimulated glucose transport may be due to not depleting glycogen enough to stimulate glucose transport. However, this seems unlikely, because our fasting regimen produced a 50% decline in glycogen in muscles from ET animals.

In conclusion, exercise training enhances the glucose transport response to insulin but inhibits the glucose transport response to hypoxia in muscles from fed ET animals. The present study also shows that elevated muscle glycogen levels are highly related to the marked reduction in hypoxia-stimulated glucose transport in muscle from ET animals. These findings demonstrate that muscle glycogen and GLUT-4 levels have little influence in regulating basal glucose transport. Furthermore, when basal glycogen values are relatively low (i.e., SED fed or semi-fasted, ET semi-fasted), maximal glycogen depletion and glucose transport stimulation are both achieved with minimal stimulus, and a relationship cannot be discerned. However, when basal glycogen levels are relatively high (i.e., ET fed), decreasing glycogen progressively stimulates glucose transport, and a strong inverse relationship exists between glycogen and hypoxia-stimulated glucose transport activity. Furthermore, when glycogen levels are high, there appears to be a threshold of glycogen depletion that is necessary for maximal stimulation of glucose transport in response to hypoxia. Finally, failure of the overexpression of GLUT-4 after exercise training to enhance the glucose transport response to contraction/ hypoxia suggests selective targeting of the additional GLUT-4 to the insulin-responsive pool.

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