Effect of carbohydrate supplementation on postexercise GLUT-4 protein expression in skeletal muscle

CHIA-HUA KUO, DESMOND G. HUNT, ZHENPING DING, AND JOHN L. IVY
Exercise Physiology and Metabolism Laboratory, Department of Kinesiology and Health Education, The University of Texas at Austin, Austin, Texas 78712

Kuo, Chia-Hua, Desmond G. Hunt, Zhenping Ding, and John L. Ivy. Effect of carbohydrate supplementation on postexercise GLUT-4 protein expression in skeletal muscle. J. Appl. Physiol. 87(6):2290–2295, 1999.—The effect of carbohydrate supplementation on skeletal muscle glucose transporter GLUT-4 protein expression was studied in fast-twitch red and white gastrocnemius muscle of Sprague-Dawley rats before and after glycogen depletion by swimming. Exercise significantly reduced fast-twitch red muscle glycogen by 50%. During a 16-h exercise recovery period, muscle glycogen returned to control levels ([25.0 ± 1.4 µmol/g] in exercise-fasted rats [24.2 ± 0.3 µmol/g]). However, when carbohydrate supplementation was provided during and immediately post-exercise by intubation, muscle glycogen increased 77% above control ([44.4 ± 2.1 µmol/g]). Exercise-fasting resulted in an 80% increase in fast-twitch red muscle GLUT-4 mRNA but only a 43% increase in GLUT-4 protein concentration. Conversely, exercise plus carbohydrate supplementation elevated fast-twitch red muscle GLUT-4 protein concentration by 88% above control, whereas GLUT-4 mRNA was increased by only 40%. Neither a 16-h fast nor carbohydrate supplementation had an effect on fast-twitch red muscle GLUT-4 protein concentration or on GLUT-4 mRNA in sedentary rats, although carbohydrate supplementation increased muscle glycogen concentration by 40% ([35.0 ± 0.9 µmol/g]). GLUT-4 protein in fast-twitch white muscle followed a pattern similar to fast-twitch red muscle. These results indicate that carbohydrate supplementation, provided with exercise, will enhance GLUT-4 protein expression by increasing translational efficiency. Conversely, postexercise fasting appears to upregulate GLUT-4 mRNA, possibly to amplify GLUT-4 protein expression on an increase in glucose availability. These regulatory mechanisms may help control muscle glucose uptake in accordance with glucose availability and protect against postexercise hypoglycemia.

Materials and Methods

Materials. GLUT-4 cDNA was generously donated by Dr. Morris J. Birnbaum (University of Pennsylvania). The 927-bp DNA fragment between EcoR I and Bgl II was gel purified as a template for production of a cDNA probe by the random priming labeling method. A 285 ribosomal RNA-specific probe was purchased from CLONTECH (Palo Alto, CA). Rat GLUT-4 antiserum was obtained from Dr. Samuel W. Cushman (NIH). Horse radish peroxidase (HRP)-conjugated donkey anti-rabbit antibody was purchased from Amersham (Arlington Heights, IL). Radioactively labeled [α-32P]CTP and [γ-32P]ATP were purchased from DuPont-New England Nuclear (Wilmington, DE). Neutral nylon membrane and random primer DNA-labeling kit were purchased from Stratagene (La Jolla, CA). Amyloglucosidase for the glycogen assay was purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals were molecular biology grade and purchased from Sigma Chemical (St. Louis, MO) and Fisher (Pittsburgh, PA).

Animals. Male Sprague-Dawley rats weighing 230–250 g were housed in a room maintained on a 0700-to-1900 light cycle and at a temperature of 21°C. The rats were allowed free access to water and chow (Purina chow, Ralston Purina, St. Louis, MO). All procedures were approved by the Animal Care and Use Committee of the University of Texas and conformed to guidelines for the use of laboratory animals published by US Department of Health and Human Services.

Experimental Procedure. Rats were randomly assigned to one of five treatment groups: exercise-fast (Ex-Fast, n = 12),
exercise-carbohydrate (Ex-Cho, n = 6), sedentary-fast (Sed-Fast, n = 6), sedentary-carbohydrate (Sed-Cho, n = 6), and sedentary-control (Sed-Con, n = 6). The exercise protocol consisted of two 3-h swimming intervals with a 45-min rest period between intervals (20). The temperature of the water was maintained between 33 and 34°C. All rats swam 10 min/day for 2 days before the start of the experiment to get familiarized with the water.

At the completion of the second 3-h swimming interval, six of the Ex-Fast rats were immediately anesthetized, and skeletal muscle was removed as described below. The remaining six Ex-Fast rats were then fasted for a 16-h period along with the Sed-Fast rats. For the Ex-Cho and Sed-Cho rats, 0.4 ml of a 50% (wt/vol) glucose solution was given by intubation at the completion of the first exercise interval and 1 ml at the completion of the second interval. In addition, rat chow was available ad libitum throughout the 16-h recovery period. During the 45-min rest period between the two swimming intervals, all treatment rats were given free access to rat chow. After the last intubation, the Ex-Cho and Ex-Sed rats were allowed to recover for 16 h like the Ex-Fast and Sed-Fast rats. For the Sed-Con rats, food was always made available, but they were not intubated with glucose. All rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (65.0 mg/kg body wt), and both gastrocnemii were quickly excised, separated into fast-twitch red and white muscle, and damped frozen with tongs cooled in liquid nitrogen. These muscle samples were used for determination of glycogen and GLUT-4 protein. Red gastrocnemius was also used for determination of GLUT-4 mRNA. Once the muscle samples were taken, the rats were killed by cardiac injection of pentobarbital sodium.

Glycogen assay. About 50 mg of muscle were dissolved in 1 N KOH at 70°C for 30 min. Dissolved homogenate was neutralized by glacial acetic acid and incubated overnight in acetate buffer (0.3 M sodium acetate, pH to 4.8) containing amyloglucosidase (4). The reaction mixture was neutralized with 1 N NaOH. Samples were then analyzed by measuring glycosyl units by the Trinder reaction (Sigma Chemical).

Measurement of GLUT-4 protein concentration. Muscle samples were homogenized (1:20, red muscle and 1:14, white muscle) in 20 mM ice-cold HEPES, 1 mM EDTA, and 0.5 M sucrose (HES) buffer (pH 7.4) with a Polytron (Brinkmann Instruments, Westbury, NY). Sample homogenates and standards were diluted 1:1 with Laemmli sample buffer (15). Muscle homogenates containing 75 µg (red gastrocnemius) or 100 µg (white gastrocnemius) of protein were then subjected to SDS-PAGE run under reducing conditions on a 12.5% resolving gel. Two GLUT-4 standards from rat heart, containing 15 and 30 µg of protein, were loaded in parallel with the muscle samples. Protein determinations were performed on each homogenate via the method of Bradford (3). Resolved proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA) as described previously (1). GLUT-4 antiserum diluted 1:500 was used for immunoblotting. GLUT-4 protein was visualized on hyperfilm by using the ECL Western blot detection kit (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions.

Measurement of GLUT-4 mRNA and ribosomal RNA concentrations. For RNA extraction, muscle tissues were homogenized in guanidium isothiocyanate-β-mercaptoethanol buffer with a Polytron. Total RNA was isolated from frozen tissue samples by the method of Chomczynski and Sacchi (5). For Northern blotting analysis, equal amounts of total RNA (30 µg) were denatured by heating at 60°C for 10 min and separated on 1% agarose-formaldehyde gels. Ethidium bromide staining of the formaldehyde gel and the transferred blots were used for determining the quality of the RNA sample. GLUT-4 mRNA was quantified by dot blotting analysis. Total RNA samples (4, 2, 1, 0.5, 0.25 µg) were applied directly to a nylon membrane by using a vacuum manifold (Bio-Rad, Richmond, CA) and were immobilized by ultraviolet cross-linking. Treatment groups were analyzed in parallel. An Escherichia coli transfer RNA was used as a control to ensure specific binding of the probe. GLUT-4 mRNA level was determined by hybridization with radioactively labeled GLUT-4 cDNA as previously described (14). All dot blots were then stripped and reprobed with 32P 5’ end-labeled gene-specific 28S ribosomal RNA oligonucleotide probe and β-actin cDNA. The amount of GLUT-4 mRNA present in each sample was determined by comparing the intensity of the sample dots with an external GLUT-4 heart standard run on each membrane.

Statistical analysis. A one-way analysis of variance among the experimental groups was performed on all variables. Fisher’s protected least significance difference test, which holds the value of a type I error to 0.05 for each test, was used to distinguish significant differences between pairs of groups. A level of P < 0.05 was set for significance for all tests, and values are expressed as means ± SE.

RESULTS

After 6 h of swimming, glycogen from both red and white sections of the gastrocnemius muscle was significantly reduced to ~50% of the preexercise level (Figs. 1 and 2). After a 16-h postexercise fast, red gastrocnemius muscle glycogen (Fig. 1) was similar to, but white...
Gastrocnemius muscle glycogen was significantly lower than sedentary control levels (Fig. 2). Exercise plus carbohydrate supplementation increased muscle glycogen above sedentary control levels by ~76% in red gastrocnemius muscle and ~42% in white gastrocnemius muscle. In contrast, carbohydrate supplementation in sedentary rats increased glycogen above sedentary control levels by only 40% in red gastrocnemius muscle and 15% in white gastrocnemius muscle. The differences in muscle glycogen between the exercised and sedentary carbohydrate-supplemented rats were significant for both red and white gastrocnemius.

Red and white gastrocnemius GLUT-4 protein concentrations were increased significantly in both the Ex-Fast and the Ex-Cho rats 16 h after exercise. In the postexercise fasted rats, red gastrocnemius GLUT-4 protein concentration increased by 43% (Fig. 3), and white gastrocnemius muscle GLUT-4 protein concentration increased by 43% (Fig. 4) above sedentary control levels. Exercise plus carbohydrate supplementation, however, increased red gastrocnemius GLUT-4 protein concentration 88% above sedentary control levels. This increase was significantly higher than that which occurred after postexercise fasting. In white gastrocnemius muscle, GLUT-4 protein concentration following exercise with carbohydrate supplementation increased 68% above sedentary control levels and 17% above the postexercise fasting level. In the sedentary rats, neither carbohydrate supplementation nor fasting significantly affected the muscle GLUT-4 protein concentration.

Irrespective of fiber type or activity status, muscle glycogen and GLUT-4 protein concentrations were found to be positively correlated 16 h after carbohydrate supplementation ($r = 0.81$) (Fig. 5). That is, the higher the GLUT-4 protein concentration of the muscle sample, the greater the glycogen concentration.

GLUT-4 mRNA concentration in red gastrocnemius muscle was significantly increased 16 h after exercise in both the carbohydrate-supplemented and fasted rats (Fig. 6). After the 16-h postexercise fasting, red gastrocnemius muscle GLUT-4 mRNA concentration increased to ~80% above sedentary control levels. In contrast, GLUT-4 mRNA was only 40% above sedentary control levels.
levels after exercise with carbohydrate supplementation. The difference in muscle GLUT-4 mRNA levels of Ex-Fast and Ex-Cho rats was significant. Neither carbohydrate supplementation nor fasting had an effect on the muscle GLUT-4 mRNA levels of sedentary rats. The concentration of $\beta$-actin mRNA in red gastrocnemius muscle across treatments was not different, indicating specificity of response for the GLUT-4 mRNA. There was also no significant difference in 28S ribosomal RNA detected among treatments (data not shown).

**DISCUSSION**

The results of this study suggest a complex interaction between exercise with carbohydrate supplementation and GLUT-4 protein expression in skeletal muscle. In agreement with previous findings, we demonstrated that an acute exercise session could initiate an increase in muscle GLUT-4 protein concentration (13, 20). In addition, we found that this exercise-induced increase in GLUT-4 protein could be enhanced by carbohydrate supplementation, although addition of the supplement resulted in a reduced muscle GLUT-4 mRNA concentration. We also observed a high correlation between the level of muscle GLUT-4 protein and glycogen concentration after a carbohydrate supplement.

The increase in GLUT-4 protein after exercise appeared to be regulated by both pretranslational and translational events. When rats were fasted for 16 h after exercise, GLUT-4 mRNA increased by 80% above control, whereas GLUT-4 protein increased only 43% above control. These results indicate that under the postexercise fasted condition muscle GLUT-4 expression was regulated by predominately pretranslational mechanisms. Furthermore, the relatively low increase in GLUT-4 protein in the presence of a relatively high GLUT-4 mRNA concentration suggests a low translational efficiency of GLUT-4 mRNA in the exercise-fasted state. In contrast to the exercise with fasting, carbohydrate supplementation plus exercise resulted in an 88% increase in GLUT-4 protein, whereas the increase in GLUT-4 mRNA was only 40%. Thus translational efficiency of GLUT-4 mRNA during the 16-h exercise-recovery period appeared to be greatly improved with carbohydrate supplementation.

In a prior study from our laboratory (13), we found that, immediately after prolonged aerobic exercise, muscle GLUT-4 mRNA was increased ~50% above control levels. When carbohydrate was provided, total GLUT-4 mRNA returned to control levels within 1.5 h and remained at this level for a minimum of 5 h. During this period, there was a substantial increase in GLUT-4 mRNA translation as GLUT-4 protein increased ~40% above the control level. An explanation for the increase in translation came in the finding that despite the decline in total GLUT-4 mRNA there was an increase in GLUT-4 mRNA-polysome association. An increase in GLUT-4 mRNA-polysome association is generally considered translationally active mRNA and therefore implies that there was an activation of the translation process during this period of recovery. The cause for this shift in translational efficiency was not evident, although it was observed to occur after the carbohydrate supplement. On the basis of the present findings, however, we can now propose that exercise plus carbohydrate supplement results in a downregulation of GLUT-4 mRNA and a conversion of the exercise-induced GLUT-4 expression from predominately pretranslational to predominately translational events.

This cellular strategy for control of GLUT-4 protein synthesis may have important physiological relevance. An increase in GLUT-4 mRNA during prolonged exer-
Exercise would prepare the muscle for rapid translation of GLUT-4 mRNA once sufficient glucose is made available for restoration of glycogen stores. The increase in GLUT-4 protein would thus promote an increase in the rate of glucose uptake and would stimulate glycogen synthesis (8, 18, 21). However, if the translational process of GLUT-4 protein was directly driven by pretranslational activation without regulation, the GLUT-4 protein production would be directly increased after the initial GLUT-4 mRNA elevation after exercise-induced glycogen depletion. Under this condition, a protracted contraction-stimulated GLUT-4 protein translocation (7, 17) would continuously forward the newly synthesized GLUT-4 protein to the plasma membrane. This could lead to a rapid clearance of blood glucose and acute hypoglycemia. Therefore, after prolonged aerobic exercise, regulation of muscle GLUT-4 protein expression at the translational level can ensure that tissues other than skeletal muscle receive appropriate amounts of glucose when blood glucose and carbohydrate reserves are low.

The mechanism by which carbohydrate supplementation provided in association with exercise downregulates GLUT-4 mRNA while simultaneously increasing GLUT-4 mRNA translation cannot be determined from the available data. However, it is possible that these processes are mediated by changes in circulating insulin levels. This possibility is supported by the observation that insulin can stimulate the downregulation of GLUT-4 mRNA in mammalian cells by inhibiting GLUT-4 gene transcription and reducing GLUT-4 mRNA stability (6). Furthermore, insulin is known to activate mRNA translation via the mitogen-activated protein kinase pathway by initiating the formation of the 40S-initiation complex by phosphorylation of the protein PHAS-1 (12, 16). Thus, during the time of GLUT-4 mRNA downregulation, the GLUT-4 mRNA in the translational cycle may be physically protected by the polysomes from the cellular degradation machinery. In contrast to the present findings, several studies have reported no change (19) or an increase (11) in GLUT-4 mRNA concentration after the infusion of glucose and insulin in sedentary rats. Thus, if insulin is involved in the downregulation of GLUT-4 mRNA as hypothesized, it may occur only in a postexercise state.

Carbohydrate supplementation had no direct effect on GLUT-4 mRNA transcription or translation. In other words, carbohydrate supplementation failed to demonstrate any changes in the levels of their muscle GLUT-4 mRNA or protein. We can thus surmise that carbohydrate supplementation exerts a modifying rather than a main stimulatory effect on the exercise-induced GLUT-4 expression. This also appears to be true for fasting animals. Sedentary rats fasted for 16 h demonstrated no changes in muscle GLUT-4 mRNA or protein. As mentioned previously, GLUT-4 mRNA was significantly higher postexercise when rats were fasted vs. when they were supplemented with carbohydrate. Thus fasting postexercise may facilitate muscle GLUT-4 mRNA transcription and thus amplify GLUT-4 mRNA translation upon an increase in glucose availability. This possibility could help explain the early observations of Bergström et al. (2) that delaying carbohydrate consumption for several days after a glycogen-depleting exercise results in greater muscle glycogen storage than starting carbohydrate replacement the day of the exercise.

Finally, the effect of carbohydrate supplementation on glycogen storage in sedentary muscle was limited compared with that of exercised muscle. With the same amount of carbohydrate supplementation, the muscle glycogen level was only increased 40% above control in sedentary rats, whereas in the exercised rats it was increased 76% above control. In addition, there was a high correlation between the level of muscle GLUT-4 protein and glycogen concentration in rats provided the carbohydrate supplement. Although prior exercise can increase the glycogen storage response to a carbohydrate supplement without an increase in GLUT-4 protein (9), it is quite possible that the level of muscle GLUT-4 protein serves to set or limit the upper level of glycogen accumulation. Thus, the difference in glycogen storage between sedentary and exercised rats may have been due in part to a difference in muscle GLUT-4 protein content. In support of this premise is the observation that glycogen storage, as well as glucose transport, is enhanced in mice genetically engineered to overexpress the GLUT-4 protein in skeletal muscle (8, 21). Furthermore, Hickner et al. (10) recently found a significant relationship between the magnitude of increase in muscle glycogen over a 48- to 72-h postexercise period and muscle GLUT-4 protein concentration when comparing trained and untrained subjects.

In summary, we have found that carbohydrate supplementation has a modifying rather than a main activating effect on exercise-induced GLUT-4 protein expression in skeletal muscle. In exercised fasted muscle, GLUT-4 mRNA was dramatically increased with less GLUT-4 protein expression compared with the exercised carbohydrate-supplemented muscle. This result suggests a possible facilitation of muscle GLUT-4 mRNA transcription during postexercise fasting. The fact that translational efficiency of GLUT-4 mRNA appears to be enhanced if carbohydrate supplement is provided with exercise, indicates that carbohydrate supplementation switches the exercise-induced GLUT-4 protein expression from a predominantly pretranslational to a predominantly translational event. The role of GLUT-4 protein expression in insulin-dependent glycogen storage in skeletal muscle has been previously suggested (13, 20). In the present study, we have shown that the degree of glycogen storage after a carbohydrate load is highly correlated with the muscle GLUT-4 protein level. This correlation suggests that the level of GLUT-4 protein expression may limit skeletal muscle glycogen storage.

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Present address of C.-H. Kuo: Taipei Physical Education College, Taipei, Taiwan.
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