Role of insulin on exercise-induced GLUT-4 protein expression and glycogen supercompensation in rat skeletal muscle

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Kuo, Chia-Hua, Hyonson Hwang, Man-Cheong Lee, Arthur L. Castle, and John L. Ivy. Role of insulin on exercise-induced GLUT-4 protein expression and glycogen supercompensation in rat skeletal muscle. J Appl Physiol 96: 621–627, 2004. First published October 10, 2003; 10.1152/japplphysiol.00830.2003.—The purpose of this study was to investigate the role of insulin on skeletal muscle GLUT-4 protein expression and glycogen storage after postexercise carbohydrate supplementation. Male Sprague-Dawley rats were randomly assigned to one of six treatment groups: sedentary control (Con), Con with streptozocin (Stz/C), immediately postexercise (Ex0), Ex0 with Stz (Stz/Ex0), 5-h postexercise (Ex5), and Ex5 with Stz (Stz/Ex5). Rats were exercised by swimming (2 bouts of 3 h) and carbohydrate supplemented immediately after each exercise session by glucose intubation (1 ml of a 50% wt/vol). Stz was administered 72 h before exercise, which resulted in hyperglycemia and elimination of the insulin response to the carbohydrate supplement. GLUT-4 protein of Ex0 rats was 30% above Con in fast-twitch (FT) red and 21% above Con in FT white muscle. In Ex5, GLUT-4 protein was 52% above Con in FT red and 47% above Con in FT white muscle. Muscle glycogen in FT red and white muscle was also increased above Con in Ex5 rats. Neither GLUT-4 protein nor muscle glycogen was increased above Con in Stz/Ex0 or Stz/Ex5 rats. GLUT-4 mRNA in FT red muscle of Ex0 rats was 61% above Con but only 33% above Con in Ex5 rats. GLUT-4 mRNA in FT red muscle of Stz/C and Stz/Ex0 rats was similar but significantly elevated in Ex5/Stz rats. These results suggest that insulin is essential for the increase in GLUT-4 protein expression following postexercise carbohydrate supplementation.

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from a tail vein and analyzed for glucose. All Stz-treated rats were confirmed as being hyperglycemic before being assigned to a treatment group. The Stz treatment was successful in ~70% of the rats. Rats not treated with Stz were injected with vehicle only.

The exercise protocol consisted of two 3-h swimming intervals with a 45-min rest between intervals (25). This protocol was selected because it had previously been found to rapidly increase the muscle GLUT-4 protein concentration (16, 17, 25). The temperature of the water was maintained between 33 and 34°C. All rats swam for 10 min/day for 2 days before the start of the experiment to familiarize them with the exercise environment. This familiarization protocol has no effect on muscle glycogen or GLUT-4 protein concentration (16).

Carbohydrate treatments were administered by oral intubation. Rats from all groups received 0.4 ml of a 50% (wt/vol) glucose solution when the first exercise interval was completed. After the second session of exercise, 1 ml of a 50% (wt/vol) glucose solution was given immediately to all rats, except for those in the Ex0 and Stz/Ex0 session of exercise, 1 ml of a 50% (wt/vol) glucose solution was given immediately to all rats, except for those in the Ex0 and Stz/Ex0 groups. Rat chow was continuously supplied ad libitum during the recovery period. Rats from the Ex0 and Stz/Ex0 groups were wrapped in a towel immediately postexercise, and a 0.5-ml blood sample was taken from a tail vein. The rats were then anesthetized in preparation for muscle sampling. Blood samples were taken from the Con, Stz/C, Ex5, and Stz/Ex5 rats 20 min after the final glucose intubation. They were then anesthetized 5 h after completion of the exercise protocol for muscle sampling. All rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (65.0 mg/kg body wt). After muscle sampling, rats were euthanized by cardiac injection of pentobarbital sodium.

Muscle sampling consisted of excising the gastrocnemius of both legs. Fast-twitch red and white sections were rapidly separated over ice and clamped frozen with tongs cooled in liquid N2. These muscle sections were used for glycogen and GLUT-4 protein determination. Fast-twitch red muscle was also used for GLUT-4 mRNA determination.

**Plasma glucose and insulin assays.** Blood samples were added to 0.1 ml EDTA (24 mg/ml, pH 7.4) and centrifuged for 10 min at 1,000 g. The plasma was recovered. Plasma glucose was measured with a glucose analyzer (Yellow Springs Instruments 23A, Yellow Springs, OH), and plasma insulin was measured by RIA (Linco, St. Charles, MO).

**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 acetic buffer (0.3 M sodium acetate, pH 4 to 4.8) containing amyloglucosidase (Boehringer Mannheim, Indianapolis, IN). The reaction mixture was neutralized with 1 N NaOH. Samples were then analyzed by measuring glucosyl units by the Trinder reaction (Sigma, St. Louis, MO).

**Glycogen synthase assay.** To best approximate the in vivo activity of glycogen synthase, its activity was determined at 30°C in the presence of 7 mmol/l ATP, 100 mmol/l UDP-1-glucose, and varying concentrations of glucose-6-phosphate (G-6-P), as described by Bloch et al. (2). Fast-twitch red gastrocnemius muscle samples were prepared for analysis by homogenization in 5 volumes of 60% glycerol, 50 mmol/l KF, and 20 mmol/l EDTA at pH 7.0 and were further diluted with 8 volumes of 30 mmol/l KF and 20 mmol/l EDTA. In addition to ATP, G-6-P, and UDP-glucose, the reaction buffer contained 50 mmol/l MOPS buffer, 25 mmol/l KF, 20 mmol/l EDTA, 10 mmol/l KH2PO4, and 10 mg/ml glycogen at pH 6.9. An eight-point dose-response curve was performed on four muscle samples from each treatment group, and nonlinear regression analysis was used to distinguish changes in sensitivity from changes in responsiveness under these conditions. Responsiveness was defined as the maximal obtainable G-6-P-stimulated reaction rate at physiological substrate and inhibitory nucleotide concentrations. Sensitivity was defined as the concentration of G-6-P at which half-maximal reaction rate occurred (A0.5). Once activity curves for glycogen synthase were constructed, the activities of glycogen synthase at the A0.5 and in the absence of G-6-P were estimated.

**Measurement of GLUT-4 protein concentration.** Muscle samples were homogenized (1:20, red muscle and 1:15, white muscle) in 20 mM ice-cold N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 1 mM EDTA, and 250 mM sucrose (hydroxylstarch) buffer (pH 7.4) with a Polytron (Brinkman Instruments, Westbury, NY). Sample homogenates and standards were diluted 1:1 with Laemmli sample buffer. Muscle homogenates containing 75 μg (red gastrocnemius) or 100 μg (white gastrocnemius) of protein were then subjected to SDS-polyacrylamide gel electrophoresis run under reduced 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 (16). GLUT-4 antiserum (a gift from Dr. Samuel W. Cushman, NIH) diluted 1:500 was used for immunoblotting. GLUT-4 protein was visualized on hyperfilm using the enhanced chemiluminescence Western Blot Detection Kit (Amer sham, Arlington Height, IL), according to the manufacturer’s instructions, and the values were made relative to the 30-μg heart standard.

**Measurement of GLUT-4 mRNA and ribosomal RNA concentration.** For RNA extraction, muscle tissues were homogenized in guaiacum 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 using a vacuum manifold (Bio-Rad, Richmond, CA) and were immobilized by ultraviolet cross-linking. Treatment groups were always 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 (a gift from Dr. Morris J. Birnbaum, University of Pennsylvania), as previously described (17). All dot blots were then stripped and reprobed with [32P]-end-labeled gene-specific 28S ribosomal RNA oligonucleotide probe and β-actin cDNA (Clontech, Palo Alto, CA). 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. 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).

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

**RESULTS**

Seventy-two hours following the Stz injection, insulin responses of Stz-treated rats to glucose intubation were effectively eliminated (Fig. 1). This resulted in all Stz rats becoming substantially hyperglycemic (Fig. 2). Con and Ex5 rats displayed plasma insulin concentrations that were six- to sevenfold higher than those of the Stz/C and Stz/Ex5 rats. Conversely, the plasma glucose concentrations of the Stz/C and Stz/Ex5 rats were four- to fivefold higher than those of the Con.
and Ex5 rats. By design, the insulin response to a glucose load could not be determined in the Ex0 or Stz/Ex0 rats, but the Stz/Ex0 rats exhibited significantly higher plasma glucose and lower plasma insulin concentrations than Ex0 rats postexercise.

Glycogen concentrations of fast-twitch red and white muscle are presented in Fig. 3, A and B, respectively. The glycogen concentration of the fast-twitch red muscle, but not the fast-twitch white muscle, of the Stz/C rats was significantly lower than that of the Con rats. Exercise resulted in significant muscle glycogen depletion in both the Ex0 and Stz/Ex0 rats. Glycogen concentrations in red and white fast-twitch muscle were similar for Ex0 and Stz/Ex0 rats. Glycogen concentrations for the Ex5 and Stz/Ex5 rats were determined 5 h after glucose intubation. For the Ex5 rats, fast-twitch red and white muscle glycogen concentrations were 35 and 30% higher than fast-twitch red and white muscle of Con rats, respectively. For the Stz/Ex5 rats, the glycogen concentration of the fast-twitch red muscle was similar to that of the Stz/C rats but significantly lower than that of the Con or Ex5 rats. The glycogen concentration of the fast-twitch white muscle showed little response to glucose intubation in the Stz/Ex5 rats and was significantly lower than that of the Con group, *P < 0.01.†Significantly different from the Con group, P < 0.01. §Significantly different from the Ex0 and Stz/Ex0 groups, P < 0.05.
lower in the Stz/Ex5 rats compared with the Con but not the Stz/C rats.

Fast-twitch red (Fig. 4A) and white (Fig. 4B) muscle GLUT-4 protein concentrations were similar for Con and Stz/C rats, respectively, suggesting that the mild Stz treatment used had no immediate effect on the constitutive expression of muscle GLUT-4 protein. Immediately after exercise, the Ex0 rats exhibited a 30% increase in fast-twitch red and a 21% increase in fast-twitch white muscle GLUT-4 protein. Five hours after glucose intubation, GLUT-4 protein concentration of Ex5 rats was 53 and 47% above Con in fast-twitch red and white muscle, respectively. GLUT-4 protein in muscle of rats exposed to Stz showed no response to exercise (Stz/Ex0) or the combination of exercise and glucose intubation (Stz/Ex5).

Table 1. Glycogen synthase activity of fast-twitch red gastrocnemius muscle determined under assay conditions that simulated normal ATP and UDP glucose cellular concentrations

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Maximal Activity, nmol g wet wt⁻¹ min⁻¹</th>
<th>A₆₅₅, mM</th>
<th>Activity at A₆₅₅</th>
<th>G-6-P-Independent Activity, nmol g wet wt⁻¹ min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>1.458±0.249</td>
<td>3.61±0.38†</td>
<td>779</td>
<td>39.5</td>
</tr>
<tr>
<td>Ex0</td>
<td>1.613±0.307</td>
<td>3.81±0.211</td>
<td>949</td>
<td>216.0</td>
</tr>
<tr>
<td>Ex5</td>
<td>1.741±0.266</td>
<td>2.99±0.09§</td>
<td>926</td>
<td>37.8</td>
</tr>
<tr>
<td>Stz/C</td>
<td>1.527±0.245</td>
<td>2.09±0.30</td>
<td>843</td>
<td>93.8</td>
</tr>
<tr>
<td>Stz/Ex0</td>
<td>1.178±0.107</td>
<td>0.82±0.16‡</td>
<td>702</td>
<td>175.0</td>
</tr>
<tr>
<td>Stz/Ex5</td>
<td>1.203±0.144</td>
<td>1.61±0.34§</td>
<td>664</td>
<td>75.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 for all groups. Con, sedentary control; Ex0, immediately postexercise; Ex5, 5-h postexercise; Stz/C, Con with a prior streptozotocin (Stz) treatment; Stz/Ex0, Ex0 with a prior Stz treatment; Stz/Ex5, Ex5 with prior Stz treatment; maximal activity, glycogen synthase activity in the presence of 33 mM glucose-6-phosphate (G-6-P); A₆₅₅, G-6-P concentration that produces half-maximal activation of glycogen synthase activity; activity at A₆₅₅, predicted activity at A₆₅₅; G-6-P-independent activity, predicted activity of G-6-P in the absence of G-6-P. †Significantly different from corresponding Stz treatment group, P < 0.05. §Significantly different from corresponding control group, P < 0.05. ‡Significantly different from corresponding postexercise group, P < 0.05.

DISCUSSION

After prolonged exercise that results in glycogen depletion, there is an increase in the muscle GLUT-4 protein concentration, but the rate of increase can be influenced by substrate availability. In this regard, our laboratory previously found that muscle GLUT-4 protein of the rat was significantly increased shortly after exercise and that the rate of increase was enhanced.
by carbohydrate supplementation (17). Muscle GLUT-4 mRNA was significantly increased immediately postexercise but rapidly returned to the preexercise level on supplementation. Muscle glycogen storage during recovery was highly correlated with the increase in GLUT-4 protein, indicating that the rate of glycogen storage after exercise is controlled, in part, by GLUT-4 protein expression. Insulin is known to be secreted on carbohydrate ingestion and to regulate both gene transcription and translation (8, 15, 18, 21). Therefore, we tested the hypothesis that the effects of carbohydrate supplementation on GLUT-4 mRNA transcription and GLUT-4 protein expression are induced by the insulin response to the supplement when provided postexercise. We also evaluated the effect of insulin on muscle glycogen storage.

To investigate the effects of insulin on GLUT-4 expression, we limited pancreatic insulin secretion by injection of Stz. The injection of Stz resulted in a severely depressed fasting insulin level and the inability of the pancreas to respond to a carbohydrate challenge. Without the normal rise in insulin following carbohydrate supplementation, the fasting blood glucose level was increased fourfold above normal. It was lowered significantly by exercise but still remained approximately threefold above Con. Blood glucose was approximately fivefold above Con 5 h after supplementation.

The inability of the pancreas to maintain a normal insulin concentration had a dramatic effect on exercise-responsive GLUT-4 protein expression. In accordance with our laboratory’s earlier research, we found that an acute bout of exercise increased the muscle GLUT-4 protein concentration. The increase in protein was significant immediately postexercise and continued to increase during the 5 h following carbohydrate supplementation. GLUT-4 mRNA was significantly increased immediately after exercise but had declined significantly by 5 h after carbohydrate supplementation. The GLUT-4 response patterns for both fast-twitch red and white muscle were similar. In contrast to the non-Stz rats, rats injected with Stz showed no increase in GLUT-4 mRNA or GLUT-4 protein immediately postexercise. During the 5 h following supplementation, there was a significant rise in GLUT-4 mRNA, but there was still no increase in GLUT-4 protein expression. These results indicate that GLUT-4 protein expression can be rapidly increased by an acute bout of exercise, but that this adaptation requires a physiological insulin concentration.

Our finding that GLUT-4 protein was increasing during the time in which GLUT-4 mRNA was declining suggests that the effect of insulin on GLUT-4 protein expression is regulated in part at the level of translation (17). Insulin has been found 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 I (18). It has also been found to downregulate GLUT-4 mRNA by inhibiting GLUT-4 gene transcription and reducing GLUT-4 mRNA stability (8), which may explain the downregulation of the GLUT-4 mRNA. However, the finding that the increase in GLUT-4 mRNA in muscle of exercised Stz-treated rats was significantly delayed suggests that insulin has at least a permissive role in the gene transcription process. This could involve the regulation or synthesis of transcription factors required for exercise-induced GLUT-4 gene transcription.

Full expression of the GLUT-4 gene appears to require the concerted interaction of the transcription factors MyoD, myocyte enhancer factor-2 (MEF-2), and the thyroid receptor-α_1 (7). Recently, Mora and Pessin (21) reported that nuclear extracts from insulin-deficient rats had reduced binding to the MEF-2 binding site compared with extracts from non-insulin-deficient rats and that insulin deficiency resulted in the down-regulation of the MEF-2 isoform MEF-2A. Replacement of MEF-2A to nuclear extracts from insulin-deficient rats fully restored binding activity of the MEF-2 element. In addition, Zheng et al. (27) reported that the adenosine analog 5-amino-imidazole-4-carboxamide ribonucleoside activates the GLUT-4 gene similar to exercise and that 5-amino-imidazole-4-carboxamide ribonucleoside activates the GLUT-4 gene. It is interesting to note that simply acutely raising plasma insulin levels by carbohydrate supplementation will not result in an increase in GLUT-4 mRNA or protein expression (17). Taken together, this information suggests that insulin deficiency may result in the downregulation of crucial transcription factors such as MEF-2A, which are essential for both exercise and hormonal regulation of GLUT-4 gene expression.

While it is likely that the repressed muscle GLUT-4 protein expression in the Stz rats was due to a reduced insulin concentration, other possibilities exist. First, the Stz rats were hyperglycemic, and, second, they were in all likelihood hyperlipemic, both of which can cause insulin resistance. However, it is unlikely that the hyperglycemia would have had an effect on GLUT-4 protein expression under the present experimental paradigm. The glucose toxicity that results from hyperglycemia requires a profuse cellular glucose uptake and, therefore, should not have had an effect under the present conditions in which plasma insulin and muscle glucose uptake were suppressed. Similarly, it is unlikely that a high plasma lipid concentration could account for the slow increase in
GLUT-4 mRNA and inability of the Stz rats to increase their muscle GLUT-4 protein levels. Animal models that are severely hyperlipidemic, such as the fatty Zucker rat (fa/fa), have normal skeletal muscle GLUT-4 protein concentrations, and their skeletal muscle GLUT-4 protein response to exercise training is normal (1, 9).

With regard to muscle glycogen, Stz treatment caused a reduction in fast-twitch red muscle glycogen but not in fast-twitch white muscle glycogen. After exercise, the difference in muscle glycogen stores between Stz-treated and nontreated rats was eliminated. In Stz-treated rats, carbohydrate supplementation was able to partially return the muscle glycogen stores back to preexercise levels, but only in the nontreated Stz rats was muscle glycogen increased above preexercise levels (glycogen supercompensation). These results agree with earlier studies in which it was reported that insulin is not required for initial restoration of skeletal muscle glycogen stores after a glycogen-depleting exercise but that it is essential for glycogen supercompensation (10, 13, 24).

After a glycogen-depleting exercise, two phases of glycogen resynthesis are typically observed in the rat (10, 13). Immediately after exercise, there is a rapid initial phase of glycogen storage to near normal levels, which can occur in the absence of insulin. This increase in glycogen storage is facilitated by an increase in membrane permeability to glucose due to a protracted increase in glucose transporters incorporated into the plasma membrane during contraction (11, 19) and activation of glycogen synthase as a result of glycogen depletion (6). Thus the increase in glycogen stores in the Stz-treated rats after exercise was likely due to the protracted increase in glucose transport and increase in glycogen synthase activity.

After the insulin-independent effect of exercise on glucose transport subsides, it is replaced by a marked increase in the sensitivity and responsiveness of muscle to insulin (10, 26). This increase in insulin action can be sustained for a prolonged period and does not seem to reverse completely until glycogen supercompensation has occurred (4, 26). The inability of Stz-treated rats to glycogen supercompensation was not due to an inability to activate muscle glycogen synthase. Postexercise glycogen synthase activity, as measured by its activity ratio, was significantly increased in both Stz-treated and nontreated rats. Furthermore, the increase in sensitivity of glycogen synthase to activation by G-6-P immediately postexercise was similar in muscle of Stz-treated and nontreated rats and was significantly higher in Stz-treated rats 5 h after carbohydrate supplementation. These results, therefore, suggest that substrate availability was rate limiting for glycogen storage in the Stz-treated rats. Because blood glucose was in excess of 400 mg% in Stz-treated rats after carbohydrate supplementation, substrate availability was likely limited by the inability of the Stz-treated rats to maintain an active muscle glucose transport after the insulin-independent phase of glycogen storage. It may also have been limited by the inability of the Stz-treated rats to increase GLUT-4 protein expression.

In the present study, we observed a rapid increase in GLUT-4 protein expression in skeletal muscle of the rat, which was insulin dependent. Insulin-stimulated muscle glucose transport is increased with increasing GLUT-4 protein concentration (12, 14), which appears to augment the rate and magnitude of glycogen storage after carbohydrate supplementation. In support of this premise is the observation that trained rats, which have an overexpression of muscle GLUT-4 protein, are more capable of storing muscle glycogen than untrained rats (22). Moreover, our laboratory previously found that 16 h after exercise-carbohydrate supplementation, compared with carbohydrate supplementation alone, muscle glycogen of the rat was significantly increased. Exercised rats were found to have a higher muscle GLUT-4 protein concentration, and this difference was found to account for 65% of the variance in glycogen storage (17). These results imply that, during an exercise-carbohydrate supplementation regimen, muscle GLUT-4 expression may directly control the magnitude of glycogen storage by elevating the glucose transport response to insulin stimulation.

In summary, the present findings indicate that the exercise-induced increase in GLUT-4 protein expression requires insulin. Insulin appears to assert its effect at the level of transcription and translation. Although insulin-deficient rats are capable of restoring their muscle glycogen stores to near normal levels in response to carbohydrate feeding postexercise, they are incapable of glycogen supercompensation. This suggests that the exercise-induced increase in GLUT-4 protein expression may be partially responsible for elevated glycogen stores that follow an exercise-carbohydrate supplementation regimen.

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GRANTS

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REFERENCES


