Control of Exercise-stimulated Muscle Glucose Uptake by GLUT4 Is Dependent on Glucose Phosphorylation Capacity in the Conscious Mouse*

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Previous work suggests that normal GLUT4 content is sufficient for increases in muscle glucose uptake (MGU) during exercise because GLUT4 overexpression does not increase exercise-stimulated MGU. Instead of glucose transport, glucose phosphorylation is a primary limitation of exercise-stimulated MGU. It was hypothesized that a partial ablation of GLUT4 would not impair exercise-stimulated MGU when glucose phosphorylation capacity is normal but would do so when glucose phosphorylation capacity was increased. Thus, C57Bl/6J mice with hexokinase II (HKII) overexpression (HKII), a GLUT4 partial knock-out (G4/−/−), or both (HKII + G4/−/−) and wild-type (WT) littermates were implanted with carotid artery and jugular vein catheters for sampling and infusions at 4 months of age. After a 7-day recovery, 5-h fasted mice remained sedentary or ran on a treadmill at 0.6 mph for 30 min (n = 9–12 per group) and received a bolus of 2-deoxy[3H]glucose to provide an index of MGU (Rg). Arterial blood glucose and plasma insulin concentrations were similar in WT, G4/−/−, HKII, and HKII + G4/−/− mice. Sedentary Rg values were the same in all genotypes in all muscles studied, confirming that glucose transport is a significant barrier to basal glucose uptake. Gastrocnemius and soleus Rg were greater in exercising compared with sedentary mice in all genotypes. During exercise, G4/−/− mice had a marked increase in blood glucose that was corrected by the addition of HK II overexpression. Exercise Rg (µmol/100g/min) was different between WT and G4/−/− mice in the gastrocnemius (24 ± 5 versus 21 ± 2) or the soleus (54 ± 6 versus 70 ± 7). In contrast, the enhanced exercise Rg observed in HKII mice compared with that in WT mice was absent in HKII + G4/−/− mice in both the gastrocnemius (39 ± 7 versus 22 ± 6) and the soleus (98 ± 13 versus 65 ± 13). Thus, glucose transport is not a significant barrier to exercise-stimulated MGU despite a 50% reduction in GLUT4 content when glucose phosphorylation capacity is normal. However, when glucose phosphorylation capacity is increased by HK II overexpression, GLUT4 availability becomes a marked limitation to exercise-stimulated MGU.

Muscle glucose uptake (MGU) can be separated into three sequential steps, i.e. delivery of glucose from the blood to the muscle, transport across the sarcolemma by a GLUT, and irreversible phosphorylation to glucose-6-phosphate by an HK isozyme. Each of these steps can serve as a barrier to MGU and, thus, are important in regulating glucose influx. During resting conditions, the transport step exerts the most control in regulating MGU, as GLUT1 (1–4) or GLUT4 (5, 6) overexpression augments basal MGU. Previous work suggests that normal GLUT4 content is sufficient for increases in MGU during exercise, because GLUT4 overexpression alone does not further increase exercise-stimulated MGU (7). Instead of glucose transport, glucose phosphorylation is a primary limitation of exercise-stimulated MGU (7–9). Heterozygous GLUT4 knock-out mice serve as a useful tool for examining the impact of reductions in glucose transport capacity on MGU (10). The aim of this study was to further define the role that GLUT4 plays in controlling MGU during exercise, when muscle glucose phosphorylation capacity is altered. It was hypothesized that a partial ablation of GLUT4 would not impair exercise-stimulated MGU because glucose phosphorylation capacity is such a marked barrier to MGU but that it do so would when glucose phosphorylation capacity is increased by HK II overexpression.

EXPERIMENTAL PROCEDURES

Mouse Maintenance and Genotyping—All procedures performed were approved by the Vanderbilt University Animal Care and Use Committee. Male FVB/NJ mice that selectively overexpress HK II (HKII) in skeletal muscle using a transgene containing the human HK II cDNA driven by the rat muscle creatine kinase promoter that yields a 3-, 5-, and 7-fold increase in HK activity in the soleus, gastrocnemius, and SVL muscles, respectively, were obtained from Dr. David E. Moller (11) and backcrossed onto the C57BL/6J background for at least seven generations. Male C57BL/6J mice with a complete ablation of the GLUT4 gene (12) were bred with HKII females to obtain heterozygous GLUT4 knock-out mice with (HKII) or without (GLUT4−/−) the HK II transgene. Mice from this mating were separated by gender following a 3-week weaning period and maintained in microisolator cages. Genotyping was performed with the polymerase chain reaction on genomic DNA obtained from a tail biopsy and isolated by a DNeasy Tissue Kit (Qiagen, Valencia, CA). All mice were fed standard

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* The abbreviations used are: MGU, muscle glucose uptake; GLUT, facilitative glucose transporter; GLUT4−/−, heterozygous GLUT4 knock-out (mouse); HK, hexokinase; HKII, HK transgene (mouse overexpressing HK II); SVL, superficial vastus lateralis; Rg, concentration-dependent index of MGU; NEFA, non-esterified fatty acid; [2-3H]DGP, 2-deoxy[3H]glucose; [2-3H]DG-6-phosphate; WT, wild type.

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Control of Exercise-stimulated Muscle Glucose Uptake in Vivo

FIG. 1. Total GLUT4 content from skeletal muscles. Immunoblotting was performed to measure total GLUT4 protein content in the gastrocnemius (B) and SVL (C) muscles of WT, GLUT4−/−, HKTg, and HKTg + GLUT4−/− mice. A representative blot is shown (A). Densitometry data are means ± S.E. for four mice per group. *p < 0.05 versus WT.

Surgical Procedures—The surgical procedures utilized are similar to those described previously (9, 13, 14). Mice were anesthetized with pentobarbital (70 mg/kg body weight). The left common carotid artery was cannulated and sealed with stainless steel plugs. Lines were flushed daily with MicroRenathane (0.033-inch outer diameter), which were exteriorized where they were attached via stainless steel connectors to lines made of PE-10 (inserted into the artery) and Silastic (0.025-inch outer diameter) tubing (22-cm long) was connected using ImageJ software (NIH).

Processing of Plasma and Muscle Samples—Immunoreactive insulin was assayed by a double antibody method (17). NEFAs were measured spectrophotometrically by an enzymatic colorimetric assay (Wako Biochemicals, Richmond, VA). Following deproteinization with Ba(OH)2 (0.3 μl) and ZnSO4 (0.3 μl), 2-[3H]DG radioactivity of the plasma was determined by liquid scintillation counting (Packard TRI-CARB 2900TR) with Ultima Gold (Packard) as the scintillant.

Muscle samples were weighed and homogenized in 0.5% perchloric acid. Homogenates were centrifuged and neutralized with KOH. One aliquot was counted directly to determine [2-3H]DG and [2-3H]DGP radioactivity. A second aliquot was treated with Ba(OH)2 and ZnSO4 to remove [2-3H]DGP and any tracer incorporated into the glycerogen and then counted to determine [2-3H]DG radioactivity. [2-3H]DGP is the difference between the two aliquots. The accumulation of [2-3H]DGP was normalized to tissue weight. The tissue-specific clearance of [2-3H]DG, or $K_p$, and the metabolic index, or $R_m$, were calculated as described previously (18) and shown in Equations 1 and 2.

![Graph A](image1.png)

**A** GLUT4 content in WT, GLUT4−/−, HKTg, and HKTg + GLUT4−/− mice. **B** HKII content in gastrocnemius and SVL muscles of WT, GLUT4−/−, HKTg, and HKTg + GLUT4−/− mice. A representative blot is shown (A). Densitometry data are means ± S.E. for four mice per group. *p < 0.05 versus WT.
GLUT4 knock-out decreased total GLUT4 content to 50% in gastrocnemius and SVL muscles studied. Muscle glycogen was lower in HK Tg mice compared with those of WT and HKTg mice throughout the 30-min sedentary period. Muscle glycogen was determined by the method of Chan and Exton.

Muscle glycogen was determined by the method of Chan and Exton (19) on the contralateral gastrocnemius and SVL muscles. Soleus glycogen could not be determined, because both of the small muscles were used to assay for [2-3H]DG and [2-3H]DP.

Statistical Analysis—Data are presented as means ± S.E. Differences between groups were determined by analysis of variance. The significance level was set at p < 0.05.

RESULTS

Descriptive Characteristics of Genetic Models—A partial GLUT4 knock-out decreased total GLUT4 content to 50% in gastrocnemius and SVL muscles as compared with WT levels (Fig. 1). Surprisingly, when combined with HK II overexpression, a disruption of a single allele of the GLUT4 gene did not markedly reduce total GLUT4 protein content in the SVL relative to HK II overexpressing mice. However, GLUT4 content in the SVL was not different between the two groups of animals with a partial GLUT4 knock-out (i.e. GLUT4−/− and HKTg + GLUT4−/−). HKII overexpression, whether alone or combined with a partial GLUT4 knock-out, increased total HKII content ~3- and ~4-fold in gastrocnemius and SVL muscles, respectively (Fig. 2).

Base-line characteristics of 5-h fasted C57BL/6J mice are shown in Table I. Deleting one GLUT4 allele, overexpressing HK II, or a combination of those two genetic manipulations did not significantly alter body weight or fasting arterial glucose and insulin concentrations. Partial GLUT4 knock-out with and without HK II overexpression reduced NEFAs in the fasted state.

Effect of Partial GLUT4 Knock-out during Rest and Exercise—GLUT4−/− mice had similar arterial glucose concentrations compared with WT mice throughout the 30-min sedentary period (Fig. 3A). GLUT4−/− mice also had similar sedentary $K_g$ (Table II) and $R_g$ values (Fig. 4) compared with WT mice in all muscles studied. However, muscle glycogen was reduced by partial GLUT4 knock-out in sedentary mice (Table III). Arterial glucose concentration tended to rise over the course of the treadmill exercise in both WT and GLUT4−/− mice (Fig. 3B). Exercise increased $K_g$ and $R_g$ significantly and decreased glycogen content in all of the muscles studied of WT and GLUT4−/− mice. A partial GLUT4 ablation did not impair $K_g$ and $R_g$ during exercise (Table II and Fig. 4, respectively). In fact, SVL $K_g$ and $R_g$ were paradoxically increased in GLUT4−/− compared with WT mice during exercise perhaps due to compensatory mechanisms. GLUT4−/− mice had lower glycogen concentrations following exercise compared with their WT littermates (Table III). However, glycogen breakdown (the difference between sedentary and exercise glycogen) was similar between WT and GLUT4−/− mice.

Effect of Partial GLUT4 Knock-out on the Background of HK II Overexpression during Rest and Exercise—HKTg + GLUT4−/− mice had similar arterial glucose concentrations as compared with those of WT and HKTg mice throughout the 30-min sedentary period (Fig. 3A). HKTg + GLUT4−/− mice had similar sedentary $K_g$ (Table II) and $R_g$ values (Fig. 4) as compared with those of WT, GLUT4−/−, and HKTg mice in all of the muscles studied. Muscle glycogen was lower in HKTg + GLUT4−/− mice as compared with that in WT, GLUT4−/−, HKTg mice in the sedentary condition (Table III).

In contrast to GLUT4−/− mice in which arterial glucose rose, HKTg + GLUT4−/− mice demonstrated a remarkably stable arterial glucose concentration during exercise (Fig. 3B). By 10 min of exercise, their glucose concentration was lower than that of GLUT4−/− mice and remained so throughout the remainder of exercise. No differences were detected compared with WT and HKTg mice. Exercise significantly increased $K_g$ and $R_g$ compared with the sedentary condition in all of the muscles.

### Table I

Baseline characteristics in 5-h fasted C57BL/6J mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GLUT4−/−</th>
<th>HKTg</th>
<th>HKTg + GLUT4−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (male/female)</td>
<td>18 (9/9)</td>
<td>18 (9/9)</td>
<td>22 (12/10)</td>
<td>18 (9/9)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>26 ± 1</td>
<td>27 ± 1</td>
<td>25 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Glucose (mg/dl−1)</td>
<td>165 ± 6</td>
<td>177 ± 9</td>
<td>170 ± 6</td>
<td>175 ± 8</td>
</tr>
<tr>
<td>Insulin (microunits/ml−1)</td>
<td>19 ± 2</td>
<td>21 ± 3</td>
<td>20 ± 3</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>NEFA (mm)</td>
<td>1.5 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

* p < 0.05 versus WT and HKTg mice.

$K_g = \frac{[2-3H]DG_{\text{muscle}}}{\text{AUC}[2-3H]DG_{\text{plasma}}}$ (Eq. 1)

$R_g = K_g \cdot \text{Glucose}_{\text{plasma}}$ (Eq. 2)

where $[2-3H]DG_{\text{muscle}}$ is the $[2-3H]DG$ radioactivity in the muscle in dpm/g, AUC[2-3H]DGplasma is the area under the plasma $[2-3H]DG$ disappearance curve calculated using the trapezoid method in dpm/mg/min, and Glucoseplasma is the average blood glucose in mm during the experimental period. $K_g$ and $R_g$ are used as concentration-independent and -dependent indices of MGU, respectively.

### Fig. 3

Arterial blood glucose during a 30-min sedentary or exercise condition in chronically catheterized conscious mice. WT, HKTg, heterozygous GLUT4−/− (●), HKTg (■), and HKTg + GLUT4−/− (○) were chronically catheterized and allowed to recover from surgery for 7 days. Following a 5-h fast, arterial blood concentration was measured during a 30-min sedentary (A) or exercise (B) condition. Data are means ± S.E. for 9–12 mice per group. * p < 0.05 for HKTg + GLUT4−/− versus GLUT4−/−.
studied of HK\textsuperscript{Tg} and HK\textsuperscript{Tg} + GLUT4\textsuperscript{+/−} mice. HK II overexpression augmented exercise-stimulated $K_g$ and $R_g$ compared with WT mice in the gastrocnemius and soleus and compared with WT mice in the SVL. This enhancement of exercise-stimulated $K_g$ and $R_g$ was abolished by partial GLUT4 knock-out in the gastrocnemius (Table II and Fig. 4A) and soleus (Table II and Fig. 4B) but not in the SVL (Table II and Fig. 4C).

Muscle glycogen was substantially lower in sedentary HK\textsuperscript{Tg} + GLUT4\textsuperscript{+/−} mice compared with other genotypes. Exercise, however, did not lead to a significant breakdown of glycogen. By the end of exercise, HK\textsuperscript{Tg} + GLUT4\textsuperscript{+/−} mice still had lower muscle glycogen compared with WT and HK\textsuperscript{Tg} mice (Table III).

**DISCUSSION**

Each step of the glucose uptake pathway (i.e. glucose delivery, transport, and phosphorylation) can be viewed as a barrier to muscle glucose influx in vivo. Mice overexpressing GLUT1 (1–4), GLUT4 (5, 6, 20–23), or HK II (1, 7, 9, 11, 24) have been used to characterize the functional sites of resistance to MGU in a variety of conditions. The logic used in these earlier studies was that if a step is a primary site of resistance to MGU, then overexpression of the protein(s) that catalyzes that step will increase MGU. An alternative approach is to increase the resistance to a step by genetically lowering the concentration or functional activity of those same proteins and determine whether such a manipulation reduces MGU. This was done for glucose phosphorylation by partially knocking out HK II (25, 26), and it was demonstrated that glucose phosphorylation is a primary site of resistance to MGU during exercise, because partial HK II knock-out mice had impaired glucose influx in oxidative muscles (26). Here we show that a partial GLUT4 ablation in C57BL/6J mice impaired neither sedentary nor exercise-stimulated MGU in any of the muscles studied at 4 months of age. Only when the barrier to glucose phosphorylation was functionally reduced by HK II overexpression could glucose transport become limiting to MGU during exercise. The model depicted in Fig. 5 demonstrates the impact of such transgenic manipulations on site-specific resistances to MGU and their outcome upon MGU.

Under basal sedentary conditions, muscle glucose transport has been viewed as the primary barrier to glucose influx due to the low concentration of glucose transporters in the sarcolemma, creating a membrane with low permeability to glucose (see Fig. 5A). This conjecture gains support from experiments in humans (27, 28) and rats (29, 30) using multiple glucose analogs and GLUT overexpression studies in mice (1–6, 20–23). The partial ablation of GLUT4, the overexpression of HK II, and the combination of the two genetic manipulations in the present study had no effect on sedentary indices of MGU (see Table III). The logic used in these earlier studies was that if a step is a primary site of resistance to MGU, then overexpression of the protein(s) that catalyzes that step will increase MGU. An alternative approach is to increase the resistance to a step by genetically lowering the concentration or functional activity of those same proteins and determine whether such a manipulation reduces MGU. This was done for glucose phosphorylation by partially knocking out HK II (25, 26), and it was demonstrated that glucose phosphorylation is a primary site of resistance to MGU during exercise, because partial HK II knock-out mice had impaired glucose influx in oxidative muscles (26). Here we show that a partial GLUT4 ablation in C57BL/6J mice impaired neither sedentary nor exercise-stimulated MGU in any of the muscles studied at 4 months of age. Only when the barrier to glucose phosphorylation was functionally reduced by HK II overexpression could glucose transport become limiting to MGU during exercise. The model depicted in Fig. 5 demonstrates the impact of such transgenic manipulations on site-specific resistances to MGU and their outcome upon MGU.

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GLUT4 by indinavir (31) reduces basal MGU; thus, some amount of GLUT4 →0 and <50% of WT levels is necessary for normal rates of basal glucose influx. Nevertheless, the results in the present study are consistent with the concept that glucose transport and not glucose phosphorylation is a key site of resistance to basal MGU.

During exercise, the massive hyperemia increases glucose delivery (32, 33), and GLUT4 translocation to the sarcolemma (34–36) increases glucose permeability. The net result of these two physiological events is that MGU markedly increases and the primary barrier to glucose influx in the working muscle shifts to glucose phosphorylation. Therefore, when HK II is overexpressed the functional resistance to glucose phosphorylation is reduced and, consequently, MGU is enhanced during exercise (see Fig. 5B). Interestingly, when total GLUT4 content is reduced by 50%, MGU is not reduced during exercise (in fact, MGU is increased in SVL). However, the enhanced exercise-stimulated $K_p$ or $R_p$ resulting from HK II overexpression is not present when combined with partial GLUT4 ablation. Thus, glucose transport becomes a key site of resistance to MGU only when glucose phosphorylation capacity is markedly increased.

Although partial ablation of GLUT4 did not alter MGU during sedentary or exercising conditions, it may reduce insulin-stimulated MGU. This has been demonstrated in isolated muscle (10, 37) and may account for the decreased muscle glycogen observed in GLUT4+/- and HK1+ + GLUT4+/- mice. The elevation in blood glucose during exercise observed in GLUT4+/- mice may compensate for any functional reductions in glucose transport. This increase in circulating glucose mass will increase the concentration gradient from the plasma to the inside of the cell and thereby increase glucose delivery and perhaps glucose transport and total glucose influx. The mechanism for this hyperglycemia is unknown but may include a yet to be discovered myokine, in this case a substance released from the muscle that signals to the liver to increase glucose production. Precedence for such interorgan communication has already been established in mice with a selective reduction of GLUT4 in adipose tissue (38). Despite reductions in glycogen content, GLUT4+/- mice had a glycogen breakdown during exercise similar to that of WT mice. HK1+ + GLUT4+/- mice, however, did not or perhaps could not mobilize glycogen during exercise, due most likely to the exceptionally low levels observed in the basal state. In a similar manner, the slightly reduced circulating NEFAs may be the result of attenuated glucose uptake by adipocytes leading to reduced precursors for fatty acid synthesis (39). The decreased reliance on fatty acids as a substrate and decreased glycogen mobilization resulting in a reduction in the glucose-6-phosphate pool, coupled with mass action effects of higher glycemia, all favor increases in MGU.

The results presented in this study are generally consistent across muscles with one exception. The exercise-stimulated $R_p$ from SVL muscles is increased in GLUT4+/- as compared with WT mice, whereas they are equal in soleus and gastrocnemius muscles. This response may be a result of the relative hyperglycemia during exercise in GLUT4+/- mice. Based on the genetic manipulation and commensurate reduction in GLUT4
protein content in this fast twitch muscle, these results are paradoxical, because reducing GLUT4 content in a muscle would be to, if anything, lower MGU during exercise. This result, however, further strengthens the general conclusions of this work, i.e. that glucose phosphorylation is the primary barrier to MGU during exercise and that the role of glucose phosphorylation in determining MGU is dependent on total GLUT4 content. Support for these conclusions is provided by the fact that HK II overexpression increases exercise-stimulated MGU in all muscles studied and that in a muscle where GLUT4 content is not markedly reduced the transgene effectiveness remains intact.

In summary, a partial ablation of GLUT4 in C57BL/6J mice did not impair sedentary or exercise-stimulated indices of MGU (i.e. $K_g$ and $R_g$). It did, however, indicate the enhanced exercise-stimulated MGU created by HK II overexpression. Therefore, despite a 50% reduction of normal GLUT4 content, glucose transport is not a significant barrier to exercise-stimulated MGU. However, a 50% reduction in GLUT4 availability makes glucose transport become a limitation to exercise-stimulated MGU when the glucose phosphorylation barrier is functionally lowered by HK II overexpression. The implication of these findings is that impairments in MGU associated with insulin resistance may not uniformly or solely reflect deficits in glucose transport capacity. These studies are consistent with the notion that increasing HK II is a reasonable therapeutic strategy for the treatment of diabetes, provided that the insulin resistance is not associated with a critically low glucose transport capacity.

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