Overexpression of Hexokinase II in Transgenic Mice

EVIDENCE THAT INCREASED PHOSPHORYLATION AUGMENTS MUSCLE GLUCOSE UPTAKE*

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Hexokinase II (HKII) is the predominant isozyme expressed in peripheral insulin-responsive tissues. To explore the role of HKII in muscle glucose metabolism, two lines of transgenic mice were generated where overexpression was restricted to striated muscle; HKII protein levels and activity were increased by 3- to 8-fold. Oral glucose tolerance, intravenous insulin tolerance, and insulin and lactate levels were unaffected in transgenic mice. Maximal insulin-stimulated glucose uptake was determined in extensor digitorum longus (soleus), and epitrochlearis muscles. Maximal insulin-stimulated glucose uptake was increased by 17% (extensor digitorum longus), 34% (soleus), and 90% (epitrochlearis) in transgenic muscles; basal and submaximal glucose uptake was also modestly increased in soleus and epitrochlearis muscles; these data suggest that increased muscle HKII (corresponding to the upper end of the physiologic range) may not be sufficient to augment net in vivo glucose homeostasis. However, glucose phosphorylation can represent a rate-limiting step for skeletal muscle glucose utilization since muscle glucose-6-phosphate levels are increased during in vivo hyperinsulinemia and hyperglycemia; furthermore, basal and insulin-mediated muscle glucose uptake can be increased by a selective increase in HKII expression.

Glucose phosphorylation to glucose-6-phosphate (G6P) by hexokinase represents the obligate first step in glucose metabolism. Of the three "low Km" mammalian hexokinases (types I, II, and III), hexokinase II (HKII) is the predominant isozyme expressed in insulin-responsive tissues (1–3).

Both HKII and the "insulin-responsive" glucose transporter GLUT4 have been suggested to be functionally paired since their levels of gene expression are coordinately regulated under a variety of physiological circumstances. During development in the rat, the appearance of both GLUT4 and HKII in skeletal muscle coincides with the acquisition of insulin sensitivity around the time of weaning (4). In adipocytes the expression of both genes is coordinately activated by insulin and repressed by insulin sensitivity (2, 5, 6). Both GLUT4 and HKII expression are also down-regulated in adipose tissue derived from insulin-resistant rodents (7). In skeletal muscle, the levels of GLUT4 and HKII vary in parallel according to fiber type, and both genes are dramatically induced by chronic electrical stimulation (8). Only a single bout of acute exercise is necessary to stimulate an increase in HKII (9) or GLUT4 (10) mRNA expression. In contrast, HKII (but not GLUT4) levels in skeletal muscle are increased by insulin infusion (5, 11) and are decreased in a model of insulin resistance (7).

Glucose transport, rather than phosphorylation, is commonly viewed as the rate-limiting step for insulin-mediated glucose metabolism in muscle (12). This hypothesis is supported by the observation that a primary increase in muscle glucose transporter expression (GLUT1 in transgenic mice) resulted in increased glucose utilization and glycogen storage (13). However, several previous studies suggest that glucose phosphorylation may be rate-limiting under certain physiologic conditions. Insulin stimulation of rat cardiac muscle (14) or isolated rat myocytes (15) results in the accumulation of intracellular free glucose, indicating that glucose phosphorylation is saturated in this setting. Accumulation of intracellular free glucose was also observed in skeletal muscle from transgenic mice overexpressing GLUT1, showing that hexokinase is saturated with the markedly increased glucose flux into this tissue (13). Furthermore, kinetic analyses of glucose uptake by the perfused rat hindlimb (16), human forearm, or in vivo glucose disposal in man (17) suggest a shift in the rate-limiting step from glucose transport to some step beyond transport under conditions of hyperinsulinemia or hyperglycemia. Finally, substantial increases in glucose transport, which occur in the context of muscle contraction, may result in glucose utilization becoming limited by phosphorylation since intracellular glucose levels are also increased by exercise (18) and electrical stimulation (19). Part of the limitation in glucose flux which is imposed by HKII during situations associated with increased glucose transport is likely to be due to feedback inhibition of the enzyme by its product, G6P (20).

Defects in insulin-mediated skeletal muscle glucose disposal represent an important component of the pathophysiology of human non-insulin-dependent diabetes mellitus. Importantly, recent evidence points to a proximal defect involving glucose
transport and its initial phosphorylation by hexokinase (21–23). A recently developed triple tracer forearm perfusion technique has allowed for the measurement of glucose phosphorylation rates in humans; in preliminary studies using this method, Pendergrass et al. (24, 25) suggest that an independent defect in muscle glucose phosphorylation exists in the muscle of non-insulin-dependent diabetes mellitus subjects or their offspring.

Given the potential importance of muscle HKII for human disease, its prominent regulation by physiologic perturbations, and controversy surrounding the question of whether phosphorylation may be rate-limiting for muscle glucose utilization, we created a new transgenic mouse model characterized by muscle-specific overexpression of human HKII. These transgenic mice were characterized by assessment of their in vivo phenotype and by determination of in vitro glucose uptake using isolated incubated muscles.

**EXPERIMENTAL PROCEDURES**

**Construction of a Human Hexokinase II Transgenic Vector**—A muscle-specific human HKII transgenic vector consisting of promoter-enhancer elements from the rat muscle creatinine kinase gene (provided by Steven Hauschka, University of Washington, and Jeff Chamberlain, University of Michigan) coupled to the human HKII cDNA and an 850-base pair cassette (SVPA) containing the polyadenylation and splice site sequences of SV40 (provided by Nadia Rosenthal, Boston University Medical Center) was generated (see Fig. 1). In brief, an EcoRI-HindIII fragment containing the full-length human HKII cDNA (3) was ligated into the pGEM vector resulting in pGEM7/HKII. The 850-base pair HindIII-BamHI SVPA cassette (26) was subcloned into pGEM11 to create pGEM11/SVPA. An 850-base pair HindII-SacI fragment of pGEM7/SVPA (containing the SVPA fragment) was then subcloned into pGEM/HKII to generate pGEM7/HKII/SVPA. A 6.5-kb KpnI-AatII fragment of the muscle creatinine kinase gene, which contains the 5′ flanking sequences, exons (non-coding), a 99-bp intron into exon 2, was used. The KpnI-AatII muscle creatinine kinase fragment was subcloned into pGEM7/HKII/SVPA to create a plasmid containing muscle creatinine kinase promoter, HKII, and SVPA. The transgene containing-fragment was then excised from the parent plasmid using AatII and MluI.

**Generation and Identification of Transgenic Mice**—Linearized transgene DNA was microinjected into male pronuclei of FVB/NJ mice and transferred to the oviducts of pseudopregnant recipient CD-1 mice as described previously (26). For Southern blot analysis of founder mice or for offspring screening, BamHI-digested genomic DNA derived from tail biopsies was used (27). Transgene integration was detected using a [32P]dCTP-labeled fragment of HKII cDNA (3). The conditions for hybridization, washing, and autoradiography have been previously described (26, 27). Transgenic mice were mated with nontransgenic FVB/NJ mice to establish hemizygous lines.

**Measurement of Circulating Glucose, Insulin, Lactate, Glucose Tolerance, and Insulin Tolerance**—Plasma insulin and glucose levels were measured with mice that were fasted overnight or fed ad libitum. Insulin and glucose were measured by standard enzymatic assay methods (Linco, St. Louis, MO). Blood glucose was measured by the glucose oxidase method as reported previously (27). Plasma lactate levels were measured using lactate dehydrogenase (Sigma, procedure 826-UV).

For glucose tolerance testing, mice were fasted overnight, anesthetized with pentobarbital (60 mg/kg body weight), and placed on a warm surface. After 30 min, basal blood samples were collected, and glucose (2 mg/kg) was administered by oral gavage. Blood samples were withdrawn at the indicated time points, and plasma glucose and insulin levels were measured as described above. For insulin tolerance tests, fasted mice were anesthetized as described above. 30 min later, basal blood samples were collected via the retro-orbital sinus, and insulin (0.4 milliunits/g) was injected via tail vein. Additional blood samples were obtained at several time points (0′, 5′, 10′, 20′, 40′). The levels of blood glucose were determined as described above.

**Measurement of Muscle Glycogen and Glucose-6-phosphate**—Muscle glycogen content was measured as we have previously described (26). For measurement of muscle G6P levels, overnight-fasted mice were anesthetized as described above, followed by administration of a mixture containing insulin (0.4 milliunits/g) and glucose (2 mg/kg) via tail vein (29). After 20 min, the gastrocnemius and heart muscles were freeze-clamped in situ, immediately excised, and stored in liquid nitrogen (30). Frozen muscle samples were weighed, pulverized, and homogenized in 10% perchloric acid (31), followed by brief centrifugation to remove insoluble matter. The supernatants were neutralized with 2 N KOH containing 0.4 M triethanolamine and 0.4 M KCl and centrifuged at 4°C. The neutralized supernatants were then used to determine G6P levels by the G6P dehydrogenase/NADP+ method (32). Sample protein concentrations were determined as described above.

**Assessment of Muscle Hexokinase II Compartmentalization**—Gluteal muscle samples were obtained from overnight-fasted mice and homogenized in a buffer containing 50 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 2 mM EDTA, 20 mM NaF. The homogenate was centrifuged at 800 × g for 45 min at 4°C. The supernatant was centrifuged at 13,000 × g for 20 min, and the resulting supernatant (S2) and pellet (P2) were subjected to measurement of HKII activity as described above. Marker enzymes were measured to confirm that the P2 fraction was enriched in mitochondrial proteins (pyruvate dehydrogenase) and that the S2 fraction was enriched in cytosolic proteins (glycogen synthase). Glycogen synthase activity was measured according to the method of Thomas et al. (33). The pyruvate dehydrogenase assay was conducted as described previously by Mandarino et al. (34).

In Vivo Muscle Incubation and Determination of 2-Deoxyglucose Uptake—Soleus, EDL, and epitrochlearis muscles were excised from mice that had been briefly anesthetized with sodium pentobarbital. Isolated muscles were preincubated for 20 min in 2 ml of incubation medium (Kreb-Henseleit buffer (KHB) containing 5 mM HEPES, 8 mM glucose, 2 mM sodium acetate, and 0.1% dialyzed bovine serum albumin (fraction V)). The muscles were then transferred to incubation medium containing 8 mM glucose, 2 μCi/ml 2-[14C]deoxyglucose, and 0.3 μCi of [14C]mannitol with 0, 0.08, or 0.10 milliunits/ml insulin followed by incubation for 30 min at 30°C while shaking and continually being gassed with 95% O2 and 5% CO2. After incubation, the muscles were rapidly blotted and freeze-clamped. Muscles were then weighed and homogenized in 1 ml of 1N KOH at 70°C for 20 min. The tissue extracts were then neutralized with 0.5 ml of 1 M HCl, and duplicate samples (0.3 ml) were transferred to vials containing 7.0 ml of BioSafeII (Research Products International Corp., Mount Prospect, IL) followed by scintillation counting after addition of 2-[14C]deoxyglucose, which is indicative of muscle glucose uptake, was initially low by subtracting the concentration of 2-[14C]deoxyglucose in the extracellular space from the total muscle 2-[14C]deoxyglucose concentration. Extracellular space was assessed by quantifying the concentration of [14C]mannitol in the muscle sample.
HKII Transgenic Mice

**RESULTS AND DISCUSSION**

A transgenic vector containing the muscle-specific promoter-enhancer region of the rat muscle creatine kinase gene (MCK) coupled to a human HKII cDNA was prepared and used for microinjection into FVB/NJ mouse zygotes. Following microinjection into fertilized FVB/NJ mouse zygotes (Fig. 1), a transgenic vector containing the muscle-specific promoter-enhancer region of the rat muscle creatine kinase gene (MCK) was used to generate independent lines (line 1, line 2) of founders. The transgenic founders were identified by Western blotting with antibodies raised against a C-terminal HKII peptide. The arrow indicates a 100-kDa protein band that corresponds to HKII. A nonspecific band of approximately 130 kDa is also evident in mouse muscle samples.

**Overexpression of human hexokinase II in skeletal muscle of transgenic mice.** For this example Western blot, 50-μg aliquots of solubilized muscle (gluteal) proteins were prepared from a control mouse or two transgenic (TG) founder mice. An additional sample of human skeletal muscle protein was included. Proteins were separated by 7% SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies raised against a C-terminal HKII peptide. The arrow indicates a 100-kDa protein band that corresponds to HKII. A nonspecific band of approximately 130 kDa is also evident in mouse muscle samples.

**Relative expression and enzyme activity of hexokinase II in different muscles derived from transgenic mice.** A, expression. Hexokinase II protein. Solubilized muscle proteins (50 μg) prepared from gluteal (Glut), quadriceps (Quad), gastrocnemius (Gast), triceps brachii (Tric), soleus, latissimus dorsi (Latis), extensor digitorum longus (EDL), diaphragm (Dia), and heart muscles were used for analysis of HKII expression by immunoblotting as described in the legend to Fig. 2. HKII protein expression was quantitated by laser densitometry. B, hexokinase II enzyme activity. Solubilized muscle proteins (10 μg) were incubated with a reaction mixture containing [14C]glucose. Labeled glucose-6-phosphate was separated by DEAE-Sephadex in the acetate form, eluted with 1 N HCl, and counted. Hexokinase II activity was calculated by subtracting heat-stable hexokinase I (=10%) from total hexokinase activity. For both sets of data, the results were expressed as fold versus mean control levels obtained with corresponding muscles obtained from non-transgenic littermate mouse. Each value shown represents the mean of data derived from three transgenic (versus three control) line 1 mice; similar results were obtained with line 2.
Whether HKII overexpression might ameliorate hyperglycemia in diabetic mice. Further studies will be required to determine glucose utilization or insulin responsiveness in otherwise normal mice. Further studies will be required to determine whether HKII overexpression might ameliorate hyperglycemia in diabetes or result in improved exercise tolerance.

Although the in vivo phenotype of HKII overexpressing mice was apparently normal, we sought to determine whether potential changes in muscle glucose uptake or metabolism could be detected. Since increased glucose flux might be expected to result in increased glycogen stores, skeletal muscle glycogen content in gluteal and gastrocnemius muscles was determined using fed transgenic mice and littermate controls. In gluteal muscle, mean glycogen levels in transgenic mice were 15.2 ± 2.2 versus 13.3 ± 1.6 μg/mg protein in controls (n = 21 mice each, not significant). There was also a nonsignificant trend toward increased glycogen in gastrocnemius muscles from transgenic mice versus controls (15.4 ± 2.3 versus 13.5 ± 1.8 μg/mg protein, n = 22 mice each). In contrast, Ren et al. (13) reported that muscle glycogen content was markedly increased in transgenic mice that overexpress GLUT1 and have a 6–7-fold increase in basal muscle glucose uptake (13).

Since G6P is the direct product of glucose phosphorylation by hexokinase, we next determined whether HKII overexpression in muscle would result in increased muscle G6P levels. Given that muscle glycogen levels were not significantly increased in transgenic mice, we attempted to accentuate potential differences by using conditions that were designed to result in elevated muscle glucose flux, which reportedly also increase muscle G6P by 60–70% (29). As shown in Fig. 5, after in vivo administration of glucose and insulin, mean G6P levels in gastrocnemius muscles from transgenic mice were 44% higher than their littermate controls. There was also a trend toward increased G6P levels in transgenic cardiac muscle (Fig. 5). The presence of higher skeletal muscle G6P concentrations in transgenic mice indicates that human HKII was functionally active in vivo. Under these experimental conditions, we also noted that the blood glucose nadir (45 min after infusion of glucose plus insulin) was lower in transgenic (2.59 ± 0.1 mM, n = 5) than in control mice (3.92 ± 0.7, n = 4, p = 0.05). In addition, recent experiments showed that in vivo 2-[3H]deoxyglucose uptake in transgenic gastrocnemius and gluteal muscles was increased relative to control during mild (230 mg/dl) hyperglycemic clamp conditions.2 Taken together, these findings are consistent with the hypothesis that, at least under conditions of hyperinsulinemia and hyperglycemia (even within the physiologic range), glucose phosphorylation is rate-limiting for muscle glucose uptake. Our results also suggest that the increase in muscle HKII expression, which follows exercise training and muscle contraction (8, 9, 38), or hyperinsulinemia (5, 11) has an important physiologic role in helping to compensate for the increased glucose transport that occurs in these settings.

To further explore the potential effects of increased muscle HKII activity, we measured basal and insulin-stimulated glu-

\[ \text{HKII Transgenic Mice} \]

\[ \text{Glucose tolerance and insulinemia in transgenic and control mice.} \]

Glucose tolerance tests (2 mg/g of body weight given by oral gavage) were performed with 8–9-week-old overnight-fasted transgenic (closed circles) and age- and sex-matched littermate control (open squares) mice. Blood samples were obtained at the indicated time points and analyzed for glucose concentration (A). Results are expressed in mmol/liter. Plasma insulin levels were also determined, and results are expressed in ng/ml (B). Each value represents the mean ± S.E. of 11–13 mice.

**Fig. 4.** Glucose tolerance and insulinemia in transgenic and control mice. Glucose tolerance tests (2 mg/g of body weight given by oral gavage) were performed with 8–9-week-old overnight-fasted transgenic (closed circles) and age- and sex-matched littermate control (open squares) mice. Blood samples were obtained at the indicated time points and analyzed for glucose concentration (A). Results are expressed in mmol/liter. Plasma insulin levels were also determined, and results are expressed in ng/ml (B). Each value represents the mean ± S.E. of 11–13 mice.

**Table I**

| Physiologic parameters | Transgenic | Control |
|------------------------|------------|---------|
| Fasted glucose (mM)    | 6.49 ± 0.5 | 6.48 ± 0.4 |
| Fed glucose (mM)       | 7.39 ± 0.3 | 7.27 ± 0.3 |
| Fasted insulin (ng/ml) | 0.71 ± 0.46 | 1.05 ± 0.25 |
| Fed insulin (ng/ml)    | 1.22 ± 0.3 | 1.25 ± 0.4 |
| Fasted lactate (mmol)  | 11.3 ± 1.0 | 10.3 ± 1.1 |
| Fed lactate (mmol)     | 30.7 ± 2.5 | 27.1 ± 2.1 |

**Fig. 5.** Effect of hexokinase II overexpression on muscle G6P levels. 8–9-week-old transgenic (TG, solid bars) or age- and sex-matched littermate control (C, hatched bars) mice were fasted overnight followed by intravenous administration of glucose (2 mg/g of body weight) and insulin (0.4 munits/mg). After 20 min, heart and gastrocnemius muscles were freeze-clamped in situ and excised. Muscle homogenates were prepared and assayed for G6P using the 6PG dehydrogenase/NADP* method as described under "Experimental Procedures." Results are expressed as nmol/g muscle protein. For gastrocnemius muscle, each point represents mean ± S.E. of data derived from 13 mice in each group. For heart, each point represents mean ± S.E. of data from 9 mice in each group. * transgenic versus control gastrocnemius muscle (p < 0.04).

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cose uptake using $2^{[3]H}$deoxyglucose as a tracer in three different muscles that were isolated from transgenic mice and their nontransgenic littermates and incubated in vitro. Results obtained with EDL, soleus, and epitrochlearis muscles from nontransgenic mice (Fig. 6) showed that the absolute rates and relative stimulation of glucose uptake by insulin were similar to data reported by others using the same mouse muscles (39). Stimulation with a maximally effective insulin concentration (10 microunits/ml) was associated with increased rates of glucose uptake in transgenic muscles that were 17% (EDL), 34% (soleus), and 90% (epitrochlearis) higher than control values. Using a submaximal insulin concentration (80 microunits/ml), we found that glucose uptake by transgenic soleus and epitrochlearis muscles was higher than in control muscles. Surprisingly, basal rates of glucose uptake were also increased in these muscles obtained from transgenic mice.

Glucose transport is generally considered to be rate-limiting for muscle glucose uptake, particularly under basal conditions or with submaximal insulin stimulation. The primary evidence that supports this contention is that levels of intracellular free glucose are thought to be very low (12, 40). However, direct measurement of intracellular glucose has not been possible (previous data represent calculated values). Furthermore, Saccomani et al. (41) performed forearm perfusion experiments in humans using a triple tracer technique and reported that only a fraction (30%) of the intracellular water space was accessible to glucose. Importantly, these investigators calculated an intracellular free glucose concentration of 1.2 mM within this space and estimated that the outside/inside glucose gradient across the plasma membrane was only 2.9 in the postabsorptive state. Thus, it is plausible that increased muscle hexokinase activity could result in an altered transmembrane glucose gradient, thereby causing increased facilitated glucose entry.

An additional potential explanation for the increased rates of glucose uptake we observed is that a secondary increase in glucose transport might exist in transgenic muscles. In a preliminary experiment, we found that net GLUT4 levels (by immunoblotting) were not elevated in skeletal muscle from HKII transgenic mice. However, the observation that acute hyperglycemia can directly promote muscle GLUT4 translocation (42) raises the possibility that slight changes in intracellular glucose metabolite concentrations could affect glucose transporter translocation or intrinsic activity. Finally, it is possible that removal of the muscles for incubation, particularly in the case of the soleus and epitrochlearis, may have caused partial activation of the glucose transport system (via a contraction-stimulated mechanism) with a shift toward glucose phosphorylation as the rate-limiting step. A comparison of data derived from hindlimb perfusion study versus isolated muscle incubations supports this hypothesis. Thus, rates of muscle glucose uptake measured during hindlimb perfusion in mice are generally lower than when the same muscles are studied in vitro incubation (43, 44). However, regardless of the mechanism, our results indicate that a selective increase in HKII activity can augment skeletal muscle glucose uptake under both submaximal and maximal stimulating conditions.

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