Regulation of Hexokinase II Gene Expression by Glucose Flux in Skeletal Muscle*

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The in vivo studies of transcriptional regulation by glucose, in general, have yielded ambiguous interpretations due to the closed loop relationship between insulin and glucose. Insulin cannot be held as a constant since elevated glucose levels will elicit a corresponding rise in insulin and current animal models of insulinopenia are associated with a plethora of counter-regulatory hormone responses. One potential solution to increase intracellular glucose flux without a further increase in insulin was achieved by transgenic overexpression of the insulin-sensitive glucose transporter, GLUT4, in specific skeletal muscles (previously described in Tsao, T.-S., Burcelin, R., Katz, E. B., Huang, L., and Charron, M. J. (1996) Diabetes 45, 28–36). Using these MLC-GLUT4 transgenic mice as a model, we investigated the effects of increased glucose flux on hexokinase II (HK II) gene expression in skeletal muscle. Under conditions where blood glucose levels were normal and insulin levels decreased by 36%, HK II mRNA level was reduced in non-GLUT4-overexpressing tissues of MLC-GLUT4 mice compared to age/sex-matched controls, and 2) GLUT4-mediated increases in cellular glucose flux can prevent the decrease in HK II expression in skeletal muscle, where overexpression of GLUT4 caused a 2.5-fold increase in basal and insulin-stimulated glucose uptake. The levels of HK II mRNA in heart, muscle, and adipose tissue are paralleled by HK II enzymatic activity. In conclusion: 1) due to relative mild insulinopenia, HK II expression is decreased in non-GLUT4-overexpressing tissues of MLC-GLUT4 mice compared to age/sex-matched controls, and 2) GLUT4-mediated increases in cellular glucose flux can prevent the decrease in HK II expression in skeletal muscle, where overexpression of GLUT4 caused a 2.5-fold increase in basal and insulin-stimulated glucose uptake.

Studies focused on the effects of glucose on gene expression in vivo are complicated by an obligatory rise in insulin subsequent to glucose administration. As a result, effects of glucose on the expression of a candidate gene cannot be distinguished from the effects of insulin (2). The in vivo feedback regulatory mechanisms can be circumvented by ex vivo studies (3). However, the results of ex vivo studies may not be as physiologically relevant as in vivo studies. With the advance of transgenic mouse technology, it is now feasible to regulate metabolic processes by varying the level of expression of genes encoding key regulatory proteins. In this report we demonstrate the utility of the transgenic approach by investigating the regulation of type II hexokinase (ATP: hexose-phosphotransferase; EC 2.7.1.1) gene expression in a line of transgenic mice that selectively overexpress GLUT4 in skeletal muscle (1).

As previously demonstrated, the MLC-GLUT4 transgenic mice overexpress GLUT4 3–4-fold specifically in skeletal muscles containing a high proportion of fast-twitch fibers (1). Overexpression of GLUT4 in skeletal muscle causes an increase in insulin-stimulated glucose transport exclusively in skeletal muscle, which in turn leads to an increase in whole body insulin action (1). The increased responsiveness to insulin results in an unique metabolic milieu in which increased glucose influx into skeletal muscle is achieved even in the presence of reduced insulin levels. In MLC-GLUT4 mice, the skewed glucose influx/insulin ratio enables one to differentiate between the effects of glucose and insulin on gene expression in skeletal muscle. Thus, MLC-GLUT4 mice represent an ideal tool with which to study the in vivo regulation of insulin-sensitive genes by glucose under conditions in which the influence of insulin is controlled.

Glucose is cleared from the circulation by its transport across the plasma membrane and subsequent phosphorylation to glucose 6-phosphate. Transport of glucose across the plasma membrane occurs by facilitated diffusion through a family of facilitative glucose transporters (4–6). A family of hexokinases phosphorylate the internalized glucose to maintain low intracellular free glucose levels and a glucose concentration gradient across plasma membrane (7). The concentration gradient ensures sustained facilitative glucose transport into a cell. The major glucose transporter and hexokinase isoforms in the insulin-sensitive tissues (skeletal muscle, heart, and adipose tissue) are GLUT4 and hexokinase II (HK II), respectively (8–10). These two proteins together constitute the first step in...
insulin-mediated glucose utilization. HK II is the second step of glucose utilization in insulin-sensitive tissues and is considered rate-limiting under conditions where glucose transport is maximally stimulated (11–13). An abundance of experimental evidence supports the hypothesis that the regulation of HK II expression or function may affect intracellular insulin action (14–16). Skeletal muscle and adipose tissue HK II mRNA levels and enzymatic activity are decreased when circulating insulin is low or when insulin signaling is impaired (10, 15, 17). The above alterations give rise to an insulin resistant state in which intracellular glucose flux is decreased. In contrast, HK II mRNA levels and enzymatic activity can be increased by exercise (18) or increased insulin-stimulated glucose influx (2). However, it is not clear whether insulin action or glucose influx alone, or the combined effect of both, is responsible for the observed modification in HK II mRNA and activity. Here we demonstrate that intracellular glucose flux has a stimulatory effect on skeletal muscle HK II gene expression which is sensitive to the effects of insulin. In addition, we show that age-induced insulin resistance in mice is associated with a decrease in steady-state HK II mRNA levels and HK II activity.

MATERIALS AND METHODS

Generation and Typing of MLC-GLUT4 Transgenic Mice—The construction of MLC-GLUT4 mice is described in detail elsewhere (1). Briefly, the 3-kilobase MLC1 f promoter (kind gift of Dr. Nadia Rosenthal) was ligated upstream of a 4.5-kilobase containing the murine GLUT4 gene, and an 847-base pair fragment end of the GLUT4 gene to confer stability to the transcript (19). The MLC enhancer was ligated at the 3′ end of the MLC-GLUT4 transgene was microinjected into the pronuclei of CBA × C57BL/6J F1 hybrids using standard techniques (21). Transgenic mice carrying the MLC-GLUT4 transgene were identified using either polymerase chain reaction or Southern blot analysis of genomic DNA extracted from tail biopsy. Animals were fed ad libitum and housed in a murine hepatitis virus-free barrier facility with the Public Health Service Animal Welfare Policy.

In Vivo 2-Deoxyglucose Uptake—For both basal and euglycemic hyperinsulinemic clamp measurements, a flash (intravenous) injection of 0.8 μCi/g of 2-[14C]deoxy-o-glucose (2-DOG, DuPont NEN) was performed after a 90-min infusion period and individual tissue glucose uptake was determined by the accumulation of [14C]2-deoxyglucose-6-phosphate (2-DOG-6-phosphate) in gastrocnemius, soleus, total hindlimb (minus soleus and part of gastrocnemius), epididymal fat, and skin 30 min after the injection. The detailed protocol for tracer infusion and euglycemic hyperinsulinemic clamp is described in Ref. 1. The specific

### Table I

| Glucose (mg/dl) | 75.1 ± 5.9 | 761 ± 3.9 | 1084 ± 10.6* | 142.8 ± 7.0 |
|----------------|------------|-----------|--------------|-------------|
| n              | 6          | 7         | 8            | 5           |

Indicates statistically significant difference (p < 0.001) between MLC-GLUT4 mice and age/sex-matched control littersmates.

### Table II

| Fasting plasma glucose and insulin of 2–4-month and 7–9-month old male MLC-GLUT4 transgenic and control mice |
|---------------------------------------------------------------|
| 2–4 month | 7–9-month |
|----------------|------------|
| Glucose (mg/dl) | 75.1 ± 5.9 | 761 ± 3.9 | 1084 ± 10.6* | 142.8 ± 7.0 |
| n              | 6          | 7         | 8            | 5           |
| Insulin (ng/ml) | 0.46 ± 0.06 | 0.72 ± 0.07 | 2.89 ± 0.55 | 2.99 ± 0.47 |
| n              | 6          | 7         | 8            | 5           |

Indicates statistically significant difference (p < 0.001) between MLC-GLUT4 mice and age/sex-matched control littersmates.

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Plasma Hormone and Metabolite Measurements—Blood was drawn from the retro-orbital sinus using a heparinized microcapillary tube and quickly spun to obtain plasma. Plasma insulin levels were measured using a rat insulin kit (Linco, Inc., St. Louis, MO). Plasma glucose was measured by the glucose oxidase (Trinder) method using a kit from Sigma Diagnostics.

Statistical Analysis—Data are presented as mean ± S.E. Statistical analysis was performed by unpaired, two-tailed Student’s t test. Differences between male MLC-GLUT4 transgenic mice and age/sex-matched control littermates were accepted as statistically significant at p < 0.05.

RESULTS

Intracellular Glucose Flux—We have measured the in vivo glucose uptake in skeletal muscle, white adipose tissue, and heart of 7–9-month-old male MLC-GLUT4 and control mice by intravenous injections of [14C]2-DOG following a 16-h overnight fast or under euglycemic hyperinsulinemic clamp conditions (1). Glucose uptake into hindlimb skeletal muscle of MLC-GLUT4 mice was 2.5-fold greater than in age-matched controls following a 16-h fast and during the euglycemic clamps (Table I). No significant difference in glucose uptake was observed in adipose tissue and heart under basal or euglycemic clamp conditions in the two experimental groups (Table I).

Plasma Insulin and Glucose—No difference in fasting plasma glucose is observed in 2–4-month-old MLC-GLUT4 mice and age/sex-matched controls (Table II). However, plasma insulin levels in MLC-GLUT4 mice are only 64% that of control mice (Table II). The plasma insulin levels of 7–9-month-old MLC-GLUT4 mice are not different from age-matched controls. In contrast, the basal plasma glucose levels of 7–9-month-old MLC-GLUT4 mice are 24% lower than that of age-matched controls. The basal plasma glucose and insulin levels of 7–9-month-old control mice are significantly elevated compared to 2–4-month-old control mice, reflecting the development of aging-associated insulin resistance (1.9- and 4.2-fold, respectively, Table II).

HK II mRNA and Activity in 2–4-Month-old Mice—As a result of the 36% decrease in basal insulin levels, HK II mRNA levels in 2–4-month-old MLC-GLUT4 mice are significantly decreased in non-transgene-overexpressing tissues such as the heart and epididymal fat (by 43 and 51%, respectively, p < 0.001, Fig. 1A). Consistent with the mRNA levels, HK II activity is decreased by 36% in the heart of 2–4-month-old MLC-GLUT4 mice (p < 0.01, Table III). No statistically significant decrease in HK II activity was observed in adipose tissue of 2–4-month-old MLC-GLUT4 mice (Table III). Interestingly, the down-regulation of HK II mRNA in the heart and adipose tissue and HK II activity in the heart was not observed in the hindlimb muscle where the MLC-GLUT4 transgene is expressed and glucose influx is increased (Fig. 1A and Table III).

HK II mRNA and Activity in 7–9-month-old Mice—Consistent with the restored basal insulin levels in 7–9-month-old MLC-GLUT4 mice, no difference in the HK II mRNA levels in the heart, adipose tissue, and hindlimb between 7–9-month-old male MLC-GLUT4 and age/sex-matched control mice was noted (Fig. 1B). In accordance with mRNA levels, no significant difference in HK II activity between 7–9-month-old MLC-GLUT4 and control mice was observed in heart, adipose tissue, and hindlimb (Table IV).

Modification of Hexokinase Isoform Expression during Aging—The HK II mRNA levels in the heart and skeletal muscle of 7–9-month-old male control mice are significantly reduced compared to 2–4-month-old male control mice (by 69.2 ± 1.8 and 73.6 ± 1.6%, respectively, Fig. 1C). In MLC-GLUT4 transgenic mice, HK II mRNA levels are decreased by 74.2 ± 3.4% in skeletal muscle and 30.4 ± 2.1% in heart during aging (Fig. 1D). However, this reduction in HK II mRNA is not significantly reflected in the HK II activity of MLC-GLUT4 or control mice between 2–4 and 7–9 months of age (Table III and IV). HK II activity in adipose tissue of MLC-GLUT4 and control mice remained unchanged with aging. In contrast to the HK II data, HK I activity was increased 33% in the heart of MLC-GLUT4 mice and 49% in the adipose tissue of control mice with aging (Table III and IV). No changes in HK I activity were noted in skeletal muscle of MLC-GLUT4 or control mice with aging (Table III and IV).

DISCUSSION

We have observed in the 2–4-month-old MLC-GLUT4 transgenic mice a decreased basal insulin level and an associated

![Table III](http://www.jbc.org/)

| Tissue distribution of hexokinase activities in 2–4-month-old MLC-GLUT4 and age/sex-matched control mice |
|---------------------------------------------------------------|
| Hexokinase activity (milliunits/mg protein)                  |
| Heart (n = 10) | Muscle (n = 10) | Fat (n = 8) | Control |
|----------------|----------------|------------|---------|
| Heart          | 113.1 ± 11.7   | 50.3 ± 3.7 | 43.3 ± 2.7 | 150.6 ± 13.1 |
| Muscle         | 49.1 ± 7.9     | 7.5 ± 1.3  | 23.4 ± 1.9 | 50.0 ± 5.1    |
| Fat            | 64.1 ± 7.5*    | 42.8 ± 3.4 | 19.9 ± 2.8 | 100.6 ± 9.9   |

* Indicates statistically significant difference (p < 0.01) between MLC-GLUT4 mice and age/sex-matched controls.
down-regulation of HK II mRNA levels in the heart and adipose tissue and decreased HK II activity in the heart. However, this down-regulation of HK II mRNA and enzyme activity was prevented in the hindlimb muscle of MLC-GLUT4 transgenic mice, where GLUT4 is overexpressed and glucose flux is increased. These results suggest that GLUT4-mediated glucose flux can increase the stimulatory effect of insulin on skeletal muscle HK II gene expression. When compared with 2-4-month-old MLC-GLUT4 mice, HK II mRNA levels are reduced in the hindlimb muscle of 7-9-month-old MLC-GLUT4 mice. Similar aging-associated reductions in HK II mRNA levels were observed in the hindlimb muscle and the heart of control mice. This reduction in HK II expression occurred despite the increase in basal insulin levels seen with aging. This may be explained by the fact that aging is associated with the developmental profile of HK II closely follows the acquisition of insulin sensitivity after weaning (16). From these previous studies it is concluded that HK II expression is acutely sensitive to age (10, 14, 17, 24). Under conditions of euglycemic fasting or streptozotocin-induced diabetes, HK II enzyme activity is reduced in skeletal muscle, and adipose tissue of rats (10, 14, 17, 24). Under conditions of euglycemic fasting or streptozotocin-induced diabetes, HK II enzyme activity is reduced in skeletal muscle, and adipose tissue of rats (10, 14, 17, 24).

The regulation of HK II gene expression by insulin is well established (28–30). Under chronic low insulin levels such as fasting or streptozotocin-induced diabetes, HK II enzyme activity is reduced in the heart, skeletal muscle, and adipose tissue of rats (10, 14, 17, 24). Under conditions of euglycemic hyperinsulinemic and hyperglycemic hyperinsulinemic clamps, HK II mRNA levels and enzymatic activity are increased in skeletal muscle and adipose tissue of rats (2). In rat, the developmental profile of HK II closely follows the acquisition of insulin sensitivity after weaning (16). From these previous studies it is concluded that HK II expression is acutely sensitive to circulating insulin levels. However, the role of glucose on HK II expression cannot be excluded since the variations in insulin levels are associated with changes in glucose flux. An interesting exception in which the level of HK II gene expression does not follow insulin levels comes from the study of the obese hyperglycemic insulin-resistant KKA Y mouse, an animal model of non-insulin-dependent diabetes mellitus (15). The levels of HK II mRNA, protein, and enzymatic activity are reduced in skeletal muscle and adipose tissue of the KKA Y mouse, yet the serum insulin levels are 30-fold greater than controls. The KKA Y mouse study indicates that sensitivity to insulin can regulate HK II gene expression independent of circulating insulin levels. This conclusion is supported by a recent study which reported that HK II activity and gene expression in skeletal muscle is reduced in patients with non-insulin-dependent diabetes mellitus (31). Our results suggest that a potential mechanism by which insulin resistance causes down-regulation of HK II is to decrease insulin-stimulated glucose uptake and flux.

The amplification effect of glucose flux on insulin-stimulated HK II gene expression in skeletal muscle may provide a regulatory mechanism by which HK II gene expression is controlled by sensitivity to insulin. In response to an increase in insulin-stimulated glucose flux, HK II mRNA and protein levels will increase to meet the demand for increased glucose flux and extend the capability of a cell to handle the increase in glucose flux by forming a positive feed-forward loop. It has been reported that glucose phosphorylation, rather than glucose transport, is rate-limiting for cellular glucose utilization when glucose transport is maximally stimulated (11–13). In a transgenic mouse model of GLUT1 overexpression in skeletal muscle, an increased glucose uptake was associated with an elevated intracellular concentration of free glucose and total hexokinase activity (11). We hypothesize that the rise in HK II levels in response to increased glucose flux should alleviate the phosphorylation of glucose as a rate-limiting step in glucose utilization.

Difficulties in separating the effects of glucose and glucose-induced insulin secretion on gene expression can be overcome by the use of cultured cells. Many ex vivo studies have shown that glucose is a transcriptional regulator (3). Studies utilizing cultured hepatocytes demonstrated that glucose stimulates the expression of L-type pyruvate kinase (32), aldolase B (33), and spot 14 gene (34). In cultured adipose cells, the expression of fatty acid synthase and acetyl-CoA carboxylase can be further enhanced by glucose or 2-deoxyglucose (35). This increase is further enhanced by insulin (35). While ex vivo studies have provided a wealth of information on the regulation of gene expression in hepatocytes and adipocytes, ex vivo muscle culture techniques have not been as fruitful. Skeletal muscle and adipose cell lines have been examined, however, they are weakly responsive to insulin (36, 37). For these reasons it is crucial to have an in vivo model such as the MLC-GLUT4 transgenic mouse to distinguish between the effects of glucose and insulin on gene expression in skeletal muscle.

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