Regulation of Hexokinase II Expression in Human Skeletal Muscle In Vivo

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The phosphorylation of glucose to glucose-6-phosphate (G-6-P) is the first committed step in glucose uptake in skeletal muscle. This reaction is catalyzed by hexokinase (HK). Two HK isoforms, HKI and HKII, are expressed in human skeletal muscle, but only HKII is regulated by insulin. The present study was undertaken to determine the time course for the regulation of HK activity and expression by physiological plasma insulin concentrations in human skeletal muscle in vivo. A hyperinsulinemic-euglycemic glucose clamp and percutaneous muscle biopsy were performed in separate groups of healthy subjects after 60, 120, 180, and 360 minutes of euglycemic hyperinsulinemia. Muscle biopsies were subfractionated into soluble and particulate fractions to determine HKI and HKII activities. RNA was extracted from a separate portion of the muscle biopsy, and HKI and HKII mRNA content was determined using an RNase protection assay. Glycogen synthase (GS) activity and fractional velocity were also determined. HKII mRNA was increased 2-fold by 120 minutes and remained high versus the basal value for up to 360 minutes. HKI mRNA was unchanged throughout the study. HKII activity increased after 360 minutes of insulin infusion, and this increase was limited to the soluble fraction. In contrast, insulin induced a 1.5- to 2-fold increase in GS fractional velocity that was sustained for 360 minutes. The time course of the ability of hyperinsulinemia to increase HKII mRNA indicates that insulin is likely a physiological regulator of HKII expression in human skeletal muscle in vivo.

INSULIN increases glucose transport in skeletal muscle by translocating glucose transporters to the plasma membrane.[1] Once glucose is transported into the cell, it is phosphorylated by hexokinase (HK) to glucose-6-phosphate (G-6-P). Of the known isoforms of HK, only HKI and HKII are expressed in skeletal muscle.[2-4] Recent evidence from studies using mathematical modeling of isotopic tracer data indicates that insulin increases glucose transport and phosphorylation independently in the forearm muscle of normal subjects, and both of these steps are resistant to insulin action in the muscle of patients with type 2 diabetes.[5,6] Insulin and muscle contraction increase the expression of HKII, but not HKI, in rodents[7,8] and humans,[2] so HKII is considered to be the isoform in skeletal muscle that is subject to insulin regulation. In normal humans, a 4-hour infusion of insulin increases HKII mRNA levels by about 3-fold in skeletal muscle, but does not affect HKI mRNA. HKII protein content increased comparably and HKII activity, partitioned subcellularly into soluble and particulate fractions, was increased in the soluble fraction.[2] HKI activity was not affected by insulin.

In a previous study,[2] a 4-hour insulin infusion was used to determine that physiological hyperinsulinemia was capable of increasing HKII expression and activity. However, 4 hours of sustained hyperinsulinemia is longer than the normal duration after the physiologic stimulus of meal ingestion in healthy humans. The most physiological experiment would be to determine the effects of a meal on HKII expression directly, but in such an experiment, it would not be possible to isolate the effects of insulin from those of meal-related hyperglycemia and other metabolic changes. Therefore, the present study was undertaken to determine whether a shorter duration of insulin infusion, more similar to the period of hyperinsulinemia observed after a meal, increases HKII mRNA levels in skeletal muscle from healthy subjects.

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SUBJECTS AND METHODS

Subjects

Nineteen lean, healthy young subjects participated in these studies. Their clinical characteristics are shown in Table 1. Routine chemistries, hematological values, and urinalysis were normal in all subjects. All subjects were instructed to avoid vigorous exercise for 48 hours before the study, and none participated in a regular conditioning program. Body weight was stable in all subjects for 3 months before the study. All subjects consumed a weight-maintaining diet containing 150 to 200 g carbohydrate for 3 days prior to the study. After obtaining informed written consent, all subjects underwent a 2-hour oral glucose tolerance test ([OGTT] 75 g) to confirm the presence of normal glucose tolerance (World Health Organization criteria). On the day of the OGTT, body composition was determined by bioelectric impedance analysis using a BIA-101Q impedance analyzer (RJL Systems, Mt. Clemens, MI). The experimental protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

Study Design

All studies began at 8 AM after a 10- to 12-hour overnight fast. Euglycemic-hyperinsulinemic (40 mU/m^2/min) clamp experiments were performed as described previously.[9] An antecubital vein was catheterized for infusion of insulin and 20% glucose, and a hand vein was cannulated in a retrograde fashion and placed in a heated box (60°C) for sampling of arterialized blood. Percutaneous needle biopsies of the vastus lateralis muscle were obtained as previously described[2] in all 19 subjects before the start of the insulin infusion and in subgroups at 60 minutes (n = 8), 120 minutes (n = 4), 180 minutes (n = 10), and 360 minutes (n = 6) during hyperinsulinemia. The second percutaneous muscle biopsy was obtained from the opposite leg. Some subjects (n = 9) had biopsies at more than one time point after the basal biopsy,

Table 1. Subject Characteristics

Characteristic	Time (min)			
	60	120	180	360
Gender (male/female)	4/4	3/1	8/2	4/2
Age (yr)	26 ± 2	25 ± 1	31 ± 3	25 ± 2
Body mass index (kg/m²)	24 ± 1	21 ± 1	24 ± 1	23 ± 1
Glucose infusion rate (mg/kg fat-free mass)	5.12 ± 0.64	7.91 ± 1.32*	8.88 ± 1.15*	10.54 ± 2.11*
Plasma insulin (μU/mL)	68 ± 7	70 ± 6	69 ± 7	66 ± 9
No. of subjects	8	4	10	6

NOTE. Data are the mean ± SEM.
*$P < .05$ *v* 60 min.

but no subject had more than 3 biopsies. Muscle biopsy specimens weighing 90 to 240 mg were immediately blotted free of blood, frozen in liquid nitrogen within 15 to 20 seconds, and stored in liquid nitrogen until analysis. After the first muscle biopsy, insulin (Novolin R; Novo Nordisk, Princeton, NJ) was infused at a rate of 40 mU/m²/min. The plasma glucose level was measured with a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA) every 5 minutes in arterialized blood, and a variable infusion of 20% glucose was adjusted to maintain euglycemia. Plasma insulin was determined by radioimmunoassay (Diagnostic Products, Los Angeles, CA).

Muscle Biopsies

For enzyme activities and immunoblots, biopsies were homogenized while still frozen using a Polytron Homogenizer (Brinkman Instruments, Westbury, CT) in a buffer consisting of 50 mmol/L potassium phosphate, pH 7.4, 2 mmol/L dithiothreitol, 2 mmol/L EDTA, 20 mmol/L sodium fluoride, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 20 μg/mL *p*-aminobenzamidine, 70 μg/mL *N*α-*p*-tosyl-L-lysine chloromethyl ketone, and 170 μg/mL phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 14,000× *g*. The supernatant (soluble fraction) was removed and kept on ice, and the pellet (particulate fraction) was resuspended in the extraction buffer containing 0.1% Triton X-100. The soluble fraction contains greater than 95% of the activity of cytosolic enzymes such as glycogen synthase (GS), and the particulate fraction contains greater than 98% of the mitochondrial enzymes such as pyruvate dehydrogenase.[10] Total RNA was extracted for RNase protection assays.[11]

Enzyme Activity Assays

The different temperature sensitivities of HKII and HKI were used to separate the activities of the 2 enzymes.[2,12] Aliquots of soluble and particulate fractions were either heated at 45°C for 1 hour or kept on ice. Because HKII activity is denatured at 45°C,[12] HK activity measured on the heated sample represents HKI activity, whereas activity measured on the samples kept at 4°C represents total HK (HKI plus HKII) activity. HKII activity was determined as the difference between these values. HK activity was determined using a modification[2] of an enzyme-linked fluorometric assay.[3] The HK assay buffer consisted of 40 mmol/L Trishydrochloride, pH 7.5, 100 mmol/L KCl, 20 mmol/L $MgCl_2$, 2 mmol/L EDTA, 10 mmol/L glucose, 2 mmol/L ATP, 0.25 mmol/L $NADP^+$, and 0.01 U/mL G-6-P dehydrogenase (Sigma Chemical, St Louis, MO). The total assay volume was 1.0 mL, and the assay was started by adding 10 μL muscle extract fractions (30 to 60 μg protein). The increase in fluorescence was monitored for 15 minutes using a fluorometer (Turner model 112; Sequoia-Turner, Mountain View, CA) calibrated to 5 nmol NADPH full-scale. This assay was linear for more than 20 minutes, during which time less than 1% of the substrates and cofactors were consumed. Multiple assays performed on separate portions of muscle biopsies from the same individuals (n = 5) showed the interassay coefficient of variation to be 11.5% ± 1.6% over a range of activity of 1 to 4 pmol · min^{-1} · μg $protein^{-1}$. GS activity was determined in cytosolic fractions using 0.1 and 10 mmol/L G-6-P as described previously.[10] GS fractional velocity was defined as the ratio of GS activities determined at 0.1 and 10 mmol/L G-6-P. This ratio is increased by insulin infusion in humans and represents dephosphorylation and activation of GS.

RNase Protection Assays for HKI and HKII mRNAs

RNase protection assays for the specific mRNA content of muscle biopsies were performed using Maxiscript and RNase Protection Assay kits (Ambion, Austin, TX). A fragment of human HKII cDNA[4] was used as a template to produce an antisense RNA probe that protects a product of 231 nt, and a fragment of human HKI cDNA[4] was used to produce a probe that protects a product of 396 nt. Two micrograms of total RNA from each biopsy was used in the protection assays. Protected products were separated by urea-polyacrylamide gel electrophoresis and quantified by PhosphorImage analysis (Molecular Dynamics, Sunnyvale, CA). A 28S rRNA probe was used as an internal control signal. In multiple experiments using aliquots of RNA from the same individuals, the intraassay coefficient of variation was determined to be about 10%. Pre- and post-insulin RNA samples were always analyzed in the same assay.

Statistical Analysis

Insulin-stimulated increments in enzyme activities and mRNA levels were compared by ANOVA (Minitab Software, State College, PA). A posteriori comparisons were performed using Student-Newman-Keuls multiple-comparison tests.

RESULTS

Euglycemic Clamp

The plasma insulin concentration and the glucose infusion rate required to maintain euglycemia during the glucose clamp

Table 2. Time Course of Insulin Effects on HKI Activity (pmol/min · μg protein)

Time of Insulin Infusion (min)	Basal		Insulin	
	Soluble	Particulate	Soluble	Particulate
60	1.98 ± 0.43	0.09 ± 0.04	2.42 ± 0.46	0.10 ± 0.03
120	2.57 ± 0.32	0.29 ± 0.10	2.97 ± 0.48	0.25 ± 0.08
180	2.11 ± 0.33	0.08 ± 0.03	1.89 ± 0.32	0.10 ± 0.03
360	2.75 ± 0.48	0.55 ± 0.26	2.87 ± 0.58	0.56 ± 0.21

NOTE. The first 2 columns depict the partitioning of HKI in each subgroup during the basal state. The last 2 columns depict the partitioning of HKI activity after 60, 120, 180, or 360 minutes of insulin infusion.

Table 3. Time Course of Insulin Effects on HKII Activity (pmol/min · μg protein)

Time of Insulin Infusion (min)	Basal		Insulin	
	Soluble	Particulate	Soluble	Particulate
60	2.38 ± 0.70	3.49 ± 0.80	2.08 ± 0.61	3.50 ± 0.79
120	2.17 ± 0.33	4.05 ± 0.43	1.88 ± 0.48	2.69 ± 0.28
180	2.92 ± 0.59	4.06 ± 0.55	3.29 ± 0.62	4.21 ± 0.64
360	1.68 ± 0.31	3.78 ± 0.45	3.04 ± 0.84*	3.53 ± 0.67

NOTE. The first 2 columns depict the partitioning of HKII in each subgroup during the basal state. The last 2 columns depict the partitioning of HKII activity after 60, 120, 180, or 360 minutes of insulin infusion.

*$P < .05$ *v* basal activities of the indicated group by paired *t* test.

are listed in Table 1. Basal plasma insulin was 6 ± 1 μU/mL, and during the euglycemic clamp, it increased to 68 ± 3 μU/mL at 60 minutes and remained constant at this level. Plasma glucose during the glucose clamp was maintained at the basal level with a coefficient of variation less than 5% in all subjects. The glucose infusion rate required to maintain euglycemia during the hyperinsulinemic-euglycemic clamp increased from 60 to 360 minutes.

Enzyme Activities

HKI and HKII activities are shown in Tables 2 and 3, respectively, and HKII is expressed as the fold-change from basal values in Fig 1A and B. Nearly all HKI activity was present in the soluble fraction and was not affected by insulin (Table 2). In contrast, HKII activity (Fig 1A) was divided between soluble (~40%) and particulate (~60%) fractions under basal conditions. After 60, 120, or 180 minutes of insulin infusion, HKII activities in the soluble and particulate fractions were not different from baseline values. After 360 minutes of insulin infusion, HKII activity in the supernatant fraction increased from 1.68 ± 0.31 to 3.04 ± 0.84 pmol/min μg (Table 3; $P < .02$). These activities at 0 and 360 minutes are shown in Fig 1B. As a marker of insulin action in muscle biopsies from these subjects, GS activity was measured. The fold-stimulation of GS fractional velocity by insulin is shown in Fig 1C and GS activity in Fig 1D. GS fractional velocity was nearly doubled

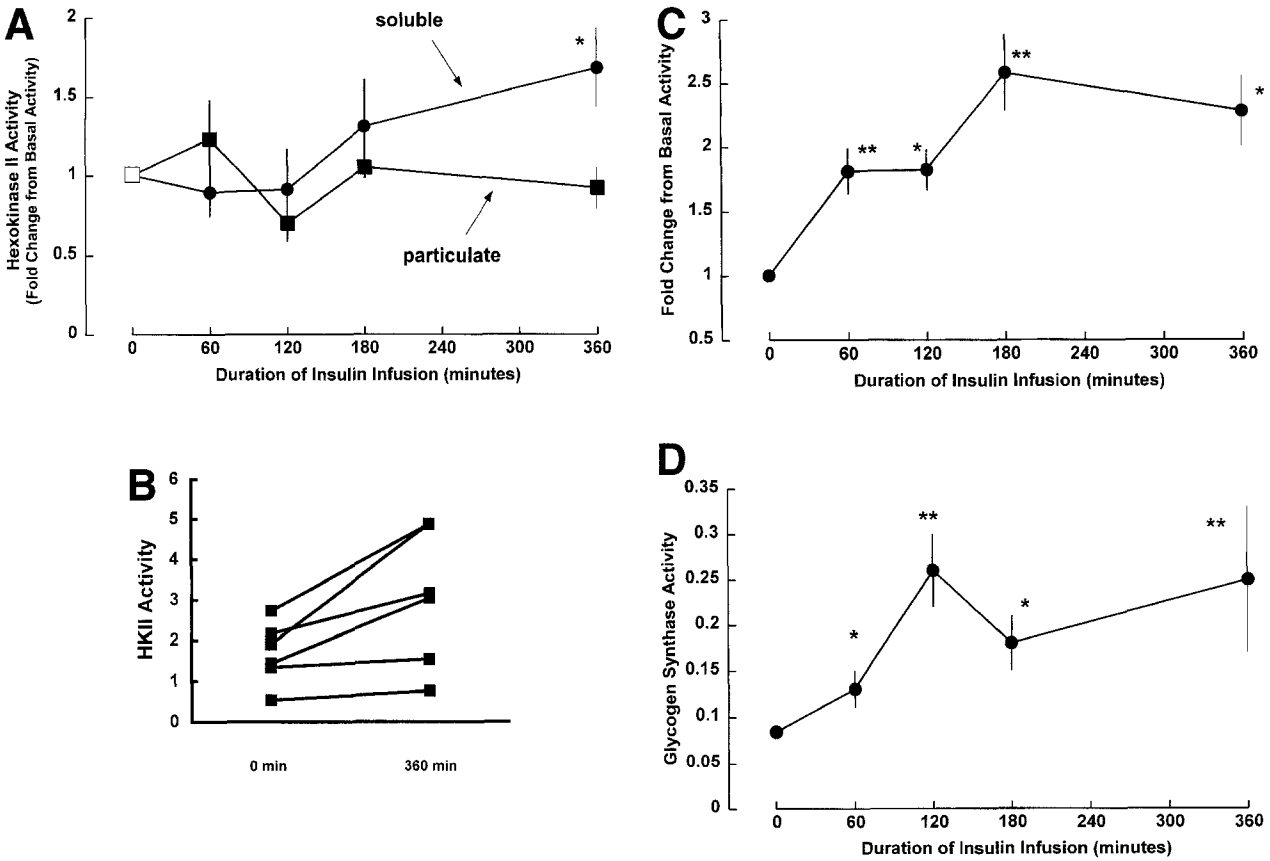

Fig 1. Time course of changes in HKII and GS activities. (A) Relative changes *v* basal values for HKII. Basal values were set to 1.0 and activities were expressed relative to this. (●) Soluble fractions; (■) particulate fractions. *$P < .05$, **$P < .01$ *v* basal value of 1.0. (B) Changes *v* basal values for HKII fold-stimulation in the 6 subjects with muscle biopsies basally and after 360 minutes of insulin infusion. Basal values were set to 1.0 and activities were expressed relative to this. HKII activities in the soluble fraction are shown. (C) Time course of stimulation of GS fractional velocity by insulin. Basal values were set to 1.0 and activities were expressed relative to this for each enzyme. *$P < .05$, **$P < .01$ *v* basal value of 1.0. (D) Time course of stimulation of GS activity. Activities were measured using 0.1 mmol/L G-6-P and are expressed as nmol/min · μg protein. The activity at time 0 is a composite average of basal values for all groups. *$P < .05$, **$P < .01$ *v* basal for the respective group. Values are the mean ± SE.

after 60 minutes of insulin infusion ($P < .01$ *v* basal), and this was maintained for up to 360 minutes of insulin infusion.

Hexokinase mRNAs

RNase protection assays were performed to determine the time course of insulin's ability to increase HKII mRNA. After 60 minutes of insulin infusion, HKII mRNA was unchanged compared with basal values, but increased at 120 minutes to a value that was 2-fold greater versus basal ($P < .05$). This increase in HKII mRNA was maintained at 180 minutes. HKII mRNA remained elevated compared with basal values after 360 minutes of insulin infusion. Insulin had no effect on HKI mRNA (Fig 2).

DISCUSSION

The present studies were undertaken to determine the time course of the insulin-stimulated increase in HKII activity and expression in skeletal muscle from healthy humans. These studies confirm that HKII, but not HKI, is regulated by insulin in skeletal muscle. Furthermore, these results show for the first time that physiological hyperinsulinemia administered over a time course similar to that found after a meal increases HKII expression in skeletal muscle. The earliest effect of insulin on HKII mRNA was observed between 60 and 120 minutes of insulin infusion.

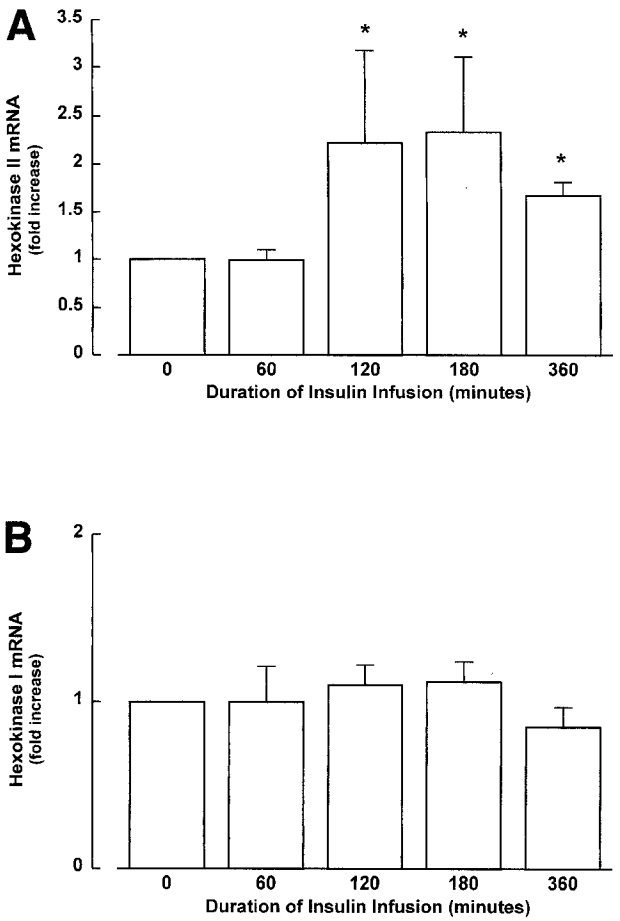

Fig 2. Effect of insulin infusion on (A) HKII and (B) HKI mRNA in muscle biopsies. Values are the mean ± SE expressed as the fold-increase over basal values set to 1.0. *$P < .05$ *v* basal (1.0).

By 2 hours, insulin doubled the content of HKII mRNA in the muscle biopsies, while HKI mRNA was unchanged. HKII mRNA remained elevated for at least 6 hours. In a previous study, it was reported that 4 hours of insulin infusion increased HKII mRNA in human skeletal muscle.[2] The current study confirms these findings and extends this result to an earlier time point that is more consistent with the duration of hyperinsulinemia after the physiological stimulus of meal ingestion. The present findings accentuate the difference in the time course of regulation of HKII expression by insulin in rat and human skeletal muscle in vivo. At least 6 hours of hyperinsulinemia was required in the rat to significantly increase HKII mRNA.[8]

It could be argued, based on the results of the previous study in human muscle,[2] that 4 hours of physiological hyperinsulinemia is a longer period of hyperinsulinemia than that to which muscle is normally exposed in healthy humans. The present finding that 2 hours of hyperinsulinemia is sufficient to increase HKII expression is more consistent with the hypothesis that insulin is a physiologically important regulator of HKII expression after a meal. Based on the present results, it can be hypothesized that the duration of hyperinsulinemia associated with meal ingestion can increase HKII expression. The time course of insulin stimulation of HKII mRNA is consistent with other reports of insulin effects on gene expression in human muscle. For example, 2 to 3 hours of hyperinsulinemia increases the level of c-*ras*, c-*myc*, c-*src*, and myf-5 mRNAs as determined using an S1 nuclease protection assay.[13]

It was previously demonstrated that HKII activity was increased after 240 minutes of insulin infusion at the same rate used in the present study.[2] In the current study, HKII activity was not increased by 180 minutes, but was significantly elevated after 360 minutes. Taken together, these data indicate that the increase in HKII mRNA at 2 hours translates to an increase in the active enzyme between 3 and 4 hours of insulin infusion. It can be speculated that newly synthesized HKII protein is targeted to the cytosolic fraction, but the present findings do not provide direct evidence of this. Previously, another report suggested that insulin infusion does not increase muscle HK activity in vivo.[14] However, in that study, no attempt was made to separate HKI and HKII activities or to subfractionate the muscle extracts into soluble and particulate fractions, so an increase in HKII activity may have been lost in a large background. Also, HKII mRNA was not measured in that study.[14] The present finding of an insulin-stimulated increase in HKII expression is consistent with data in vivo in rats[11] or in cultured L6 rat skeletal muscle cells.[3] Nevertheless, these findings point out that both insulin-sensitive and -insensitive isoforms of HK are abundantly expressed in human skeletal muscle. Their specific roles in the regulation of glucose uptake remain to be defined.

The influence of particular steps in glucose metabolism on the overall rate of glucose uptake into skeletal muscle has been controversial. The well-known correlation of insulin stimulation of GS activity with glucose disposal promoted the idea that this enzyme has a major role in determining the rate of glucose uptake.[10,15-17] These findings are supported by the observation that transgenic mice overexpressing GS have increased glucose

uptake.[18] However, more recent studies using nuclear magnetic resonance spectroscopy or mathematical modeling of isotopic tracer washout curves have implicated more proximal steps, either glucose transport or phosphorylation, in regulating the rate of glucose uptake.[6,19] In the present study, the rate of glucose uptake as indicated by the glucose infusion rate required to maintain euglycemia continued to increase between 1 and 6 hours of insulin infusion, over the same period when HKII mRNA led to increased HKII activity. These results do not prove that HKII activity regulates the rate of glucose uptake, but they demonstrate that HKII expression and insulin-stimulated glucose uptake parallel one another. This is consistent with the possibility that HKII activity has a regulatory role in glucose uptake and is not merely a passive partner.

With regard to the role of glucose transporters in the regulation of glucose uptake, glucose transport and phosphorylation are coupled metabolically and are often thought to be regulated together. However, previous studies have dissociated the regulation of these two first steps in glucose uptake.[2,8] Although HKII mRNA, protein, and activity were increased in response to insulin infusion in healthy subjects[2] or in rats,[8] GLUT4 mRNA and protein levels were unchanged. So in this case, it would seem that insulin-induced changes in HKII expression and activity are more closely related to changes in glucose uptake than is GLUT4 expression. However, it is possible, even likely, that alterations in GLUT4 translocation are more important for determining the rate of insulin-stimulated glucose uptake than even GLUT4 protein content. The assessment of the rate of glucose transporter translocation in vivo in humans is still problematic.

GS fractional velocity, which reflects the phosphorylation state of the enzyme,[20] was also measured in the soluble fraction of the muscle biopsies. In contrast to the more chronic effect of insulin on HKII expression, insulin maximally activated GS within 60 minutes. The time course of insulin's activation of GS has previously been reported,[21] and it was shown previously that 4 hours of hyperinsulinemia does not increase GS expression.[2] The present findings accentuate the contrast between insulin's acute and more chronic effects.

In summary, the present study demonstrates that insulin increases the expression of HKII mRNA within 2 hours, which is well within the physiological period of hyperinsulinemia following a meal. The increase in mRNA translated to an increase in HKII activity between 3 and 4 hours. These results are consistent with a physiological role for the regulation of HKII mRNA by insulin in muscle from healthy humans.

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