Impaired Activity and Gene Expression of Hexokinase II in Muscle from Non-Insulin-dependent Diabetes Mellitus Patients

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Abstract

After entering the muscle cell, glucose is immediately and irreversibly phosphorylated to glucose-6-phosphate by hexokinases (HK) I and II. Previous studies in rodents have shown that HKII may be the dominant HK in skeletal muscle. Reduced insulin-stimulated glucose uptake and reduced glucose-6-phosphate concentrations in muscle have been found in non-insulin-dependent diabetes mellitus (NIDDM) patients when examined during a hyperglycemic hyperinsulinemic clamp. These finding are consistent with a defect in glucose transport and/or phosphorylation. In the present study comprising 29 NIDDM patients and 25 matched controls, we tested the hypothesis that HKII activity and gene expression are impaired in vastus lateralis muscle of NIDDM patients when examined in the fasting state. HKII activity in a supernatant of muscle extract accounted for 28±5% in NIDDM patients and 40±5% in controls ($P = 0.08$) of total muscle HK activity when measured at a glucose media of 0.11 mmol/liter and 31±4 and 47±7% ($P = 0.02$) when measured at 11.0 mmol/liter of glucose. HKII mRNA, HKII immunoreactive protein level, and HKII activity were significantly decreased in NIDDM patients ($P < 0.0001$, $P = 0.03$, and $P = 0.02$, respectively) together with significantly decreased glycogen synthase mRNA level and total glycogen synthase activity ($P = 0.02$ and $P = 0.02$, respectively). In the entire study population HKII activity estimated at 0.11 and 11.0 mM glucose was inversely correlated with fasting plasma glucose concentrations ($r = -0.45$, $P = 0.004$; $r = -0.54$, $P < 0.0001$, respectively) and fasting plasma nonesterified fatty acid concentrations ($r = -0.46$, $P = 0.003$; $r = -0.37$, $P = 0.02$, respectively). In conclusion, NIDDM patients are characterized by a reduced activity and a reduced gene expression of HKII in muscle which may be secondary to the metabolic perturbations. HKII contributes with about one-third of total HK activity in a supernatant of human vastus lateralis muscle. (*J. Clin. Invest.* 1995. 96:2639–2645.) Key words: muscle hexokinase II gene expression • non-insulin-dependent diabetes mellitus • insulin resistance

Introduction

Four isoenzymes of hexokinase (HKI–IV)[1] are known to exist in mammalian tissues (1). Hexokinases I, II, and III share several properties including a molecular mass of ~ 100 kD, a high affinity for glucose, and inhibition by the reaction product glucose-6-phosphate (G6P) (2). HKIV, commonly referred to as glucokinase, has a molecular mass of 50 kD and is primarily located in liver and pancreatic β cells, has a lower affinity for glucose, and is not subject to feedback regulation by G6P (3).

From previous studies in rodents it has been shown that HKII predominates in muscle tissue where it is found in particular in a mitochondrial-free, sarcoplasmatic vesicle-rich fraction and it has been proposed that HKII may function in muscle mainly to aid glycogen production (1, 4). Moreover, studies of streptozotocin diabetic rats have shown decreased activity of gastrocnemius muscle HKII, possibly due to a loss in the amount of HKII protein rather than to an inhibition by G6P or other metabolites (5). Furthermore, within 2 h after insulin administration to diabetic rats net HK activity was restored to normal values, suggesting that insulin could be involved in the regulation of HKII synthesis (5).

Under clamp conditions of euglycemia and hyperinsulinemia, glucose clearance in patients with non-insulin-dependent diabetes mellitus (NIDDM) is characteristically decreased by 30–50% with glucose storage being the quantitatively most affected pathway (6). Muscle glucose transport and glycogen synthesis are both insulin-stimulated pathways and each has separately been suggested to be responsible for the reduced rate of insulin-stimulated glucose disposal in NIDDM patients. Thus, impaired insulin-stimulated 3-*O*-methylglucose transport in muscle strips isolated from NIDDM patients has been reported (7, 8). Furthermore, studies in healthy volunteers and insulin-dependent diabetic subjects show that glucose transport in muscle may be rate-limiting for overall glucose disposal over a wide range of plasma insulin and glucose levels (9, 10). In patients with long-term NIDDM, in vivo measurement of the muscle content of G6P by ^{31}P nuclear magnetic resonance during a hyperglycemic hyperinsulinemic clamp showed a decrease in both gastrocnemius muscle content of G6P and in nonoxidative glucose metabolism when compared with healthy control subjects. Although these studies measured G6P concentration rather than flux through the G6P pool, these findings may be consistent with a defect in glucose transport and/or phosphorylation inducing a secondary reduction in the rate of muscle glycogen synthesis (11). Moreover, using a novel triple tracer technique in obese diabetic and nondiabetic patients, it has been demonstrated that transmembrane glucose transport and intracellular glucose phosphorylation in vivo are similarly impaired in skeletal muscle and may contribute to the insulin resistance

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1. *Abbreviations used in this paper:* G6P, glucose-6-phosphate; GS, glycogen synthase; HK, hexokinase; NEFA, nonesterified fatty acids; NIDDM, non-insulin-dependent diabetes mellitus.

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Table I. Clinical Characteristics of Participants

	Control	NIDDM
Sex (F:M)	9:16	11:18
Age (yr)	53 (40–65)	55 (43–64)
Duration of diabetes (yr)	—	4.9±0.9
Body mass index ($kg \cdot m^{-2}$)	27±1	28±1
HbA_{1C} (%)	5.3±0.1	7.9±0.3*
Fasting plasma glucose (mM)	5.2±0.1	11.7±0.7*
Fasting serum insulin (pM)	52±7	68±8
Fasting serum C-peptide (nM)	0.55±0.04	0.66±0.05
Fasting plasma NEFA (mM)	0.48±0.05	0.78±0.06‡
Fasting plasma triglycerides (mM)	1.27±0.18	2.60±0.53§

Means±SE. * $P < 0.0001$; ‡ $P = 0.0006$; § $P = 0.002$.

found in these patients when compared with lean nondiabetic control subjects (12). However, the latter suggestions are challenged by the results from two other studies (13, 14). When NIDDM patients and matched control subjects were examined during similar glucose utilization rates and similar circulating insulin levels, NIDDM patients had an increased intracellular concentration of free glucose and G6P in muscle tissue biopsies, indicating that the rate-limiting step in muscle glucose metabolism in patients with NIDDM under these experimental conditions may be located after G6P.

Recently, a cDNA encoding human skeletal muscle HKII was cloned and sequenced (15, 16). The polypeptide encoded by the HKII gene comprises 917 amino acids. Based on the predicted amino acid sequence we have raised a polyclonal antipeptide antibody against the enzyme. Hence, to gain further insight into the abnormal glucose metabolism of skeletal muscle from patients with NIDDM, we have examined during basal conditions the activities of HK and the gene expression of HKII in NIDDM patients and matched healthy control subjects.

Methods

Subjects. 29 patients with NIDDM and 25 normal subjects participated in the study. Subgroups of both NIDDM patients and controls have been studied before (17). During a hyperinsulinemic euglycemic clamp NIDDM patients were shown to be insulin resistant when compared with matched controls (17). All were Caucasians and sedentary. The control subjects had normal fasting plasma glucose, normal blood pressure, and no family history of diabetes or other known insulin-resistant states. Clinical data are presented in Table I. Individuals with NIDDM as defined by the National Diabetes Data Group (18) were recruited from the outpatient clinic at Steno Diabetes Center. Only NIDDM patients with a fasting serum C-peptide level > 0.3 nmol/liter were included in the protocol. 13 patients were on sulfonylurea therapy alone or in combination with metformin (4 patients), 1 patient received metformin alone, and 15 were on weight-maintaining diet therapy alone. The diets typically contained 45% carbohydrate, 40% fat, and 15% protein. No significant differences in clinical data (Table I) were seen between the group of NIDDM patients which was treated by diet alone and the group of patients which was treated with drugs. Before participation the purpose and risks of the study were carefully explained to all of the volunteers and their informed consent was obtained. The protocol was approved by the local Committee of Ethics in Copenhagen and was in accordance with the Helsinki Declaration.

Study protocol. All experiments were started in the fasting state at 0800 in the morning after a 10-h overnight fast. A venous blood sample was drawn and concentrations of plasma glucose, serum insulin, serum C-peptide, plasma nonesterified fatty acids (NEFA), plasma triglycerides, and HbA_{1C} were measured. Percutaneous muscle biopsies (~ 400 mg) were obtained under local anesthesia (1% lidocaine without epinephrine) from the vastus lateralis muscle ~ 20 cm above the knee, using a modified Bergström needle (Stille-Werner, Copenhagen, Denmark). Muscle samples were blotted to remove blood and connective and adipose tissue, and were within 30 s frozen in liquid nitrogen and stored at −80°C until assayed.

Determination of glycogen synthase (GS) and HK activities. Extraction of muscle samples (~ 50 mg), measurement of protein, and analysis of GS activity were performed as described previously (19).

One aliquot of supernatant of muscle extracts was assayed in duplicate spectrophotometrically for total HK activity (HKI and HKII) in the presence of two concentrations of glucose (0.11 and 11.0 mmol/liter) by a modification of the method of Easterby and Qadri (20). The same initial supernatant used for the GS assay was used for HK assay except without dilution. Both for the GS and HK assays, Triton X-100 was included as a detergent to assay full enzyme activity. The supernatant was assayed at 22°C in a buffer containing (final concentrations) 28 mmol/liter Tris-HCl (pH 7.6), 17.9 mmol/liter $MgCl_2$, 2.8 mmol/liter EDTA, 0.9 mmol/liter $NADP^+$, 0.6 mmol/liter ATP, and 13.8 μg/ml G6P dehydrogenase (Boehringer Mannheim, Mannheim, Germany). The G6P produced in the HK reaction is coupled to G6P dehydrogenase. The reaction is followed by measuring the increase in absorbance at 340 nm due to NADPH formation. Another aliquot of supernatant from NIDDM patients and control subjects was heated for 60 min at 45°C in the absence of glucose and then assayed to assess heat-stable HK activity. Heat treatment results in the loss of ~ 95% of HKII activity, whereas HKI activity is reduced by only 10% (2). In heat-treated samples, therefore, HKI activity is predominant. HKII activity was estimated as the difference between total HK and HKI activity.

Total HK, HKI, and HKII activities were expressed as nanomoles of glucose converted per minute per milligram of extracted protein.

Interassay coefficient of variation for total HK activity was 0.10 (n = 6 separate experiments performed on muscle tissue from the same volunteer).

To exclude any influence of day-to-day variation, muscle samples from NIDDM patients and control subjects were prepared and analyzed for HK and GS enzyme activities pairwise at the same time.

Preparation of HKII antipeptide antibody. A peptide containing the nine-amino acid HKII carboxy terminus [(NHN_2)-CRIREAGQR-COOH] was synthesized (Kem-En-Tec, Copenhagen, Denmark) and coupled to keyhole limpet hemocyanin using glutaraldehyde. 1.4 ml 0.4% (vol/vol) glutaraldehyde (G 5882; Sigma Immunochemicals, St. Louis, MO) in 0.01 M phosphate buffer, pH 7.2, was added dropwise to a mixture of 4 mg peptide and 4 mg keyhole limpet hemocyanin (H-2133; Sigma Immunochemicals) in 0.8 ml 0.01 M phosphate buffer, pH 7.2. Stirring was continued overnight at 4°C. The product was dialyzed overnight against 0.1 M phosphate buffer, pH 7.2, then overnight against 0.01 M glycine buffer, pH 7.2, and then finally overnight against 0.01 M phosphate buffer, 0.15 M NaCl buffer, pH 7.2. Precipitation occurred during the last dialysis step. Rabbits were immunized with subcutaneous injections on days 0, 14, 28, 56, 140, and 224 with 0.5 mg conjugate contained in a volume of 0.5 ml mixed with 0.5 ml adjuvant. The first injection was made with Freund's complete adjuvant and the following with Freund's incomplete adjuvant. The animals were bled on days 35, 62, and then regularly every month.

Immunoblotting. 15–20 mg of frozen muscle (1 mg tissue/20 μl buffer) was homogenized at 4°C in a buffer, pH 7.4, consisting of 25 mM Hepes, 10 mM K-EDTA, 100 mM NaF, 1% (vol/vol) Triton X-100, and 1 mM benzamidine, 900,000 kU/liter Trasylol (aprotinin), and 2 mM PMSF and centrifuged at 2,000 rpm for 45 s. The protein concentration was determined in triplicates on the supernatant by the method of Lowry et al. (21).

Samples, 150 μg of protein per lane, were separated on an 8% SDS-PAGE gel with a 4% stacking gel. A human muscle protein standard preparation was run on each gel in triplicate and used to correct for intragel variations in the immunoblots. Biotinylated molecular weight markers were from Bio Rad Labs (Hercules, CA). The proteins were electrophoretically transferred to a 0.45-μm nitrocellulose membrane

(200 V, 2 h at 0–4°C, Bio-Rad high efficiency transfer system with plate electrons). After blocking with 5% (wt/vol) bovine serum albumin, 20 mM Tris, 500 mM NaCl, pH 7.5, for 12–24 h the membranes were incubated with HKII antiserum (1:100) for 12–24 h at 4°C and, after washing, detection of the immunocomplex with horseradish peroxidase–labeled anti–rabbit IgG antibody (ECL; Amersham International, Buckinghamshire, United Kingdom) was carried out. Quantitative scanning of autoradiograms was performed within the linear response range, as determined by standard curves of HKII protein, using software from a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Studies of the immunoreactivity of HKII in muscle specimens from NIDDM patients and controls were run in parallel at the same time. When seven different samples from the same muscle biopsy were prepared separately, the interassay coefficient of variation was 0.04 for immunoreactive HKII protein.

Coincubation with HKII antiserum and the peptides which were used to immunize the rabbits resulted in complete disappearance of the band at ~ 100 kD.

RNA extraction. Muscle biopsies (50 mg) were homogenized in a 4 M guanidinium thiocyanate solution and subsequently total RNA was isolated on a nucleic acid purification system (model 341; Applied Biosystems, Inc., Foster City, CA). Quantity and purity of total RNA were determined by absorbance at 260 and 280 nm.

Measurement of skeletal muscle HKII and GS mRNAs by PCR. The levels of HKII and GS mRNAs in total RNA from skeletal muscle biopsies were measured using a PCR method as described previously (22), except that the results were corrected for β-actin levels as measured by the same method. Briefly, HKII, GS, and β-actin mRNA were each reverse-transcribed and coamplified (28, 17, and 19 PCR cycles for HKII, GS, and β-actin, respectively), with a rabbit β-globin standard (0.5 μg of total RNA was spiked with 0.01, 1.25, and 1.25 pg rabbit globin mRNA [Clontech, Palo Alto, CA] for HKII, GS, and β-actin, respectively). One primer in each primer set was fluorescein labeled (f) which allowed for determination of the level of HKII, GS, or β-actin PCR products relative to the β-globin standard PCR products. The PCR products were analyzed on an automated laser fluorescence DNA sequencer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

The primers used were: HKII: 5′-acaggtgctctcaagccctaag-3′ and 5′-fcgaggccgcatctcagagcgg-3′; GS: 5′-acctggcttattcccaactgctc-3′ and 5′-fagtgacctcaggttctggatcatg-3′; β-actin: 5′-fggttccgctgccctgaggcac-3′ and 5′-cactgtgttggcgtagaggtc-3′; β-globin: 5′-tttggcaaagaattcactcctcag-3′ and 5′-fgatgctcaaggggcttcatgatg-3′.

The competitive reverse transcription-PCR method applied here to measure HKII mRNA levels in total RNA samples isolated from muscle biopsies has been validated in three ways. First, a sample of total RNA isolated from human muscle has been included and measured five times in each experiment performed ($n = 3$). The SE of this test sample was < 10%. Second, this test sample was also used to determine the number of PCR cycles necessary to achieve a reproducible signal (28 cycles for HKII). Importantly, this sample did not change the ratio of β-globin standard when analyzed after 31 PCR cycles. This sample had an arbitrary level for HKII of 88 before correction with β-actin and 146 after correction with β-actin. Finally, several of the tested samples, in the present study, had arbitrary HKII levels near 300 after corrections with β-actin, thus showing that the method is capable of measuring high values.

Other analytical procedures. Glucose in plasma was measured by an automated glucose oxidase method (Granutest; Merck, Darmstadt, Germany). Serum insulin and C-peptide concentrations were analyzed by a two-site sandwich ELISA method and RIA, respectively (23–25). HbA_{1C} was measured by a HPLC method, normal range 4.1–6.1%. Plasma triglycerides were analyzed using a standard method. Free fatty acids (NEFA) in plasma were determined by the method of Itaya and Michio (26).

Statistical analysis. Statistical analysis was performed with the SPSS package (Statistical package for the social sciences; SPSS Inc., Chicago, IL). Nonparametric statistics were used: Mann-Whitney test for unpaired data and Wilcoxon test for paired data and Spearman's test for correlation analysis. P values < 0.05 were considered significant. All data in text and figures are given as mean±SE.

Table II. Basal HK Activities in Vastus Lateralis Muscle from Control Subjects and NIDDM Patients

	Control	NIDDM
	nmol/mg protein/min	
Total HK activity		
0.11 mmol/liter glucose	4.8±0.2 (n = 16)	4.2±0.2* (n = 24)
11.0 mmol/liter glucose	6.7±0.4 (n = 16)	5.8±0.2‡ (n = 24)
HKI activity		
0.11 mmol/liter glucose	2.8±0.2 (n = 16)	3.1±0.2 (n = 24)
11.0 mmol/liter glucose	3.5±0.3 (n = 16)	4.0±0.3 (n = 24)
HKII activity		
0.11 mmol/liter glucose	2.0±0.2 (n = 16)	1.2±0.2‡ (n = 24)
11.0 mmol/liter glucose	3.2±0.4 (n = 16)	1.8±0.2§ (n = 24)

Means±SE. NIDDM group versus control group. * $P = 0.06$; ‡ $P = 0.02$; § $P = 0.004$.

Results

HK activity. Total HK as well as HKI activities were measured in muscle biopsies from the study participants. Muscle HKII activity was assessed as the difference between total and HKI activity. Using this approach we found that HKII activity was significantly decreased in NIDDM patients as assessed at both low (0.11 mmol/liter) and high (11.0 mmol/liter) glucose levels ($P = 0.02$ and 0.004, respectively) (Table II). This difference was a cause of decreased total HK activity in the NIDDM patients (0.11 mmol/liter glucose: 4.2±0.2 vs. 4.8±0.2 nmol/mg protein/min [$P = 0.06$]; 11.0 mmol/liter glucose: 5.8±0.2 vs. 6.7±0.3 nmol/mg protein/min [$P = 0.02$], NIDDM vs. control subjects) whereas no significant differences were found in HKI activity (0.11 mmol/liter glucose: 3.1±0.2 vs. 2.8±0.2 nmol/mg protein/min [NS]; 11.0 mmol/liter glucose: 4.0±0.3 vs. 3.5±0.3 nmol/mg protein/min [NS], NIDDM vs. control subjects). In muscle from NIDDM patients HKII represents 29±5% of total HK activity whereas in muscle from the control subjects HKII represents 41±5% ($P = 0.08$) at the low glucose level. At the highest glucose level comparable data were found, 31±4 and 47±7% ($P = 0.02$), NIDDM and control subjects, respectively. The increase in HK activity from 0.11 to 11.0 mmol/liter glucose was significant in NIDDM and control subjects for total HK, HKI, and HKII activities ($P < 0.005$ for all). However, the glucose-induced activation of HKII was significantly higher in the control subjects ($P = 0.02$) and a similar tendency to higher activation of HKI was also seen in the control group ($P = 0.08$).

HKII activity as assessed at both low and high glucose levels was inversely correlated to both fasting plasma glucose ($r = -0.45$, $P = 0.004$ and $r = -0.54$, $P < 0.0001$) (Fig. 1), plasma NEFA ($r = -0.46$, $P = 0.003$ and $r = -0.37$, $P = 0.02$) (Fig. 2) and HbA_{1C} ($r = -0.33$, $P = 0.03$ and $r = -0.54$, $P < 0.0001$) when estimated in the total group of study subjects ($n = 40$).

No differences were found in the total protein content of muscle homogenates between the groups in the basal period (78.0±4.4 vs. 76.9±2.3 mg/gram wet weight of muscle tissue [NS], NIDDM vs. control, respectively).

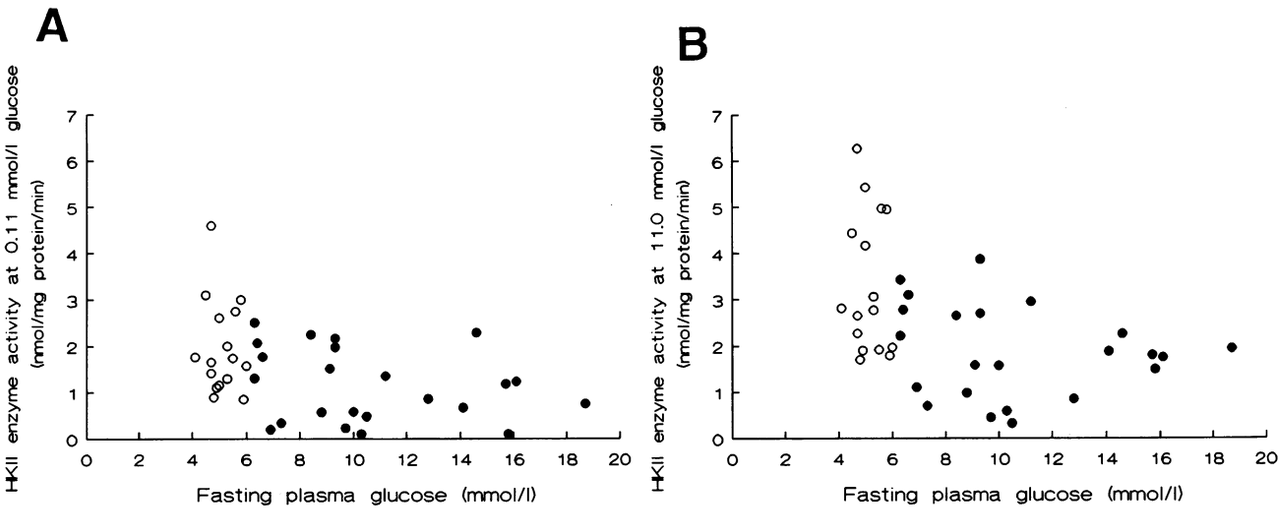

Figure 1. Muscle HKII activity as assessed at both (*A*) low (0.11 mmol/liter) and (*B*) high (11.0 mmol/liter) glucose levels in the assay was inversely correlated to fasting plasma glucose ($r = -0.45$, $P = 0.004$ and $r = -0.54$, $P < 0.0001$, respectively) in the entire group of study participants ($n = 40$). NIDDM: $r = -0.31$, $P = 014$ (low glucose), and $r = -0.36$, $P = 0.08$ (high glucose); controls: $r = -0.17$, $P = 0.52$ (low glucose), and $r = -0.18$, $P = 0.49$ (high glucose). *Filled circles,* NIDDM; *open circles,* control.

GS activity. Total GS activity (i.e., in the presence of a saturating concentration of the allosteric activator G6P) was significantly higher in the group of control subjects ($n = 20$) as compared with the group of NIDDM patients ($n = 23$) when measured at the fasting serum insulin level (29.6±1.2 vs. 34.1±1.5 nmol/mg protein/min, $P = 0.005$, NIDDM vs. control, respectively).

HKII mRNA levels. The abundance of the specific HKII mRNA relative to human β-actin mRNA showed a four- to fivefold variation in vastus lateralis muscle within the groups of participants. In the basal state, HKII mRNA was significantly decreased in the NIDDM patients when compared with the control subjects (55±7 [$n = 20$] vs. 162±20 [$n = 20$] arbitrary units [$P < 0.0001$]) (Fig. 3).

HKII mRNA levels were inversely correlated to both fasting plasma glucose ($r = -0.73$, $P < 0.0001$) (Fig. 4 *A*), plasma NEFA ($r = -0.60$, $P < 0.0001$) (Fig. 4 *B*), and HbA_{1c} ($r = -0.54$, $P < 0.0001$) when estimated in the entire group of study participants (Fig. 4, *A* and *B*) ($n = 40$).

GS mRNA levels. Using the PCR technique, the abundance of GS mRNA relative to human β-actin mRNA was assessed from muscle biopsies taken in the basal period in a subgroup of 14 NIDDM patients and 14 control subjects. The GS mRNA was significantly decreased in the NIDDM patients (69±6 vs.

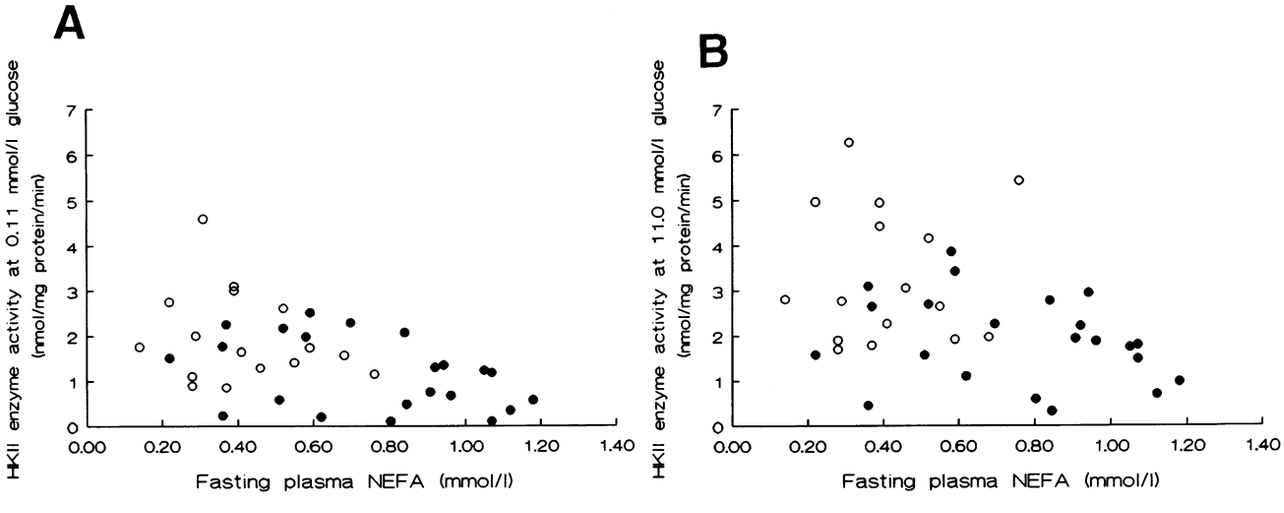

Figure 2. Muscle HKII activity as assessed at both (*A*) low (0.11 mmol/liter) and (*B*) high (11.0 mmol/liter) glucose levels in the assay was inversely correlated to fasting plasma NEFA ($r = -0.46$, $P = 0.003$ and $r = -0.37$, $P = 0.02$, respectively) in the entire group of study participants ($n = 40$). NIDDM: $r = -0.39$, $P = 0.06$ (low glucose), and $r = -0.35$, $P = 0.09$ (high glucose); controls: $r = -0.14$, $P = 0.62$ (low glucose), and $r = 0.13$, $P = 0.63$ (high glucose). *Filled circles,* NIDDM; *open circles,* control.

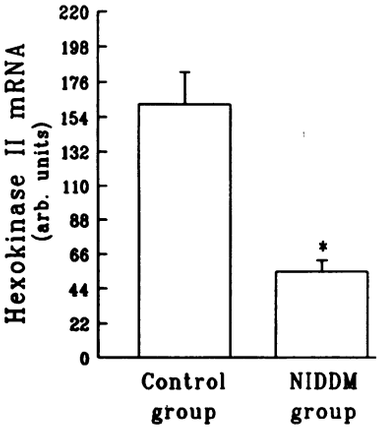

Figure 3. The abundance of HKII mRNA levels in skeletal muscle from NIDDM patients (n = 20) and healthy control subjects (n = 20) relative to human β-actin mRNA was assessed using the PCR technique as described in Methods. Basal HKII mRNA was significantly decreased in NIDDM patients (*P < 0.0001). Values are mean±SE.

85±4 arbitrary units [P = 0.02], NIDDM vs. control, respectively).

HKII immunoreactive protein levels. The amount of HKII immunoreactivity in homogenates from vastus lateralis muscle was quantitated by immunoblotting using antipeptide antiserum specific for HKII. In all participants a dominant band of ~ 100 kD was identified for HKII immunoreactive protein (Fig. 5 *A*).

Densitometric scanning of autoradiograms showed significantly decreased levels of HKII protein in the entire group of NIDDM patients compared with the control group when results were normalized for equal amounts of protein (77±6 [n = 28 NIDDM patients] vs. 96±5 OD unit/150 μg protein [percentage of internal standard] [n = 25 control subjects] [P = 0.03] [Fig. 5 *B*]).

Expressing HKII enzyme activities in the groups of study participants relative to the HKII immunoreactive protein levels we found no differences in HKII activity between the NIDDM and control subjects, indicating that the decreased HKII enzyme activity in NIDDM patients may be due to a reduced level of HKII protein (data not shown).

Discussion

HKI and HKII, the two HK isoforms present in skeletal muscle, can be partially distinguished based on their differential heat stabilities. Incubation at 45°C for 60 min in the absence of glucose reduces HKI and HKII activity by ~ 10 and ~ 95%, respectively (2). In heat-treated samples, therefore, HKI is the predominant HK activity. Using this approach in supernatants of muscle homogenates from both NIDDM and control subjects examined in the fasting state we found that heath-stable HKI activity is normal in NIDDM whereas HKI activity represents a significantly higher percentage of total HK activity in NIDDM patients (~ 72%) when compared with the control subjects (~ 60%). This is clearly higher than what has been reported in diabetic and control rat (27, 28) and diabetic and control mouse (1, 29) skeletal muscle. However, previous studies using electrophoretic methods to assess the amount of specific HK proteins have found more HKI than HKII protein in human skeletal muscle (30, 31), thus indirectly confirming our results.

In the NIDDM patients we found a significantly decreased muscle HKII activity. Cloning and sequencing of the HKII cDNA from human skeletal muscle (15, 32) made it possible to examine whether these functional abnormalities in glucose metabolism were accompanied by alterations in the pretranslational regulation of HKII. The level of basal HKII mRNA from skeletal muscle biopsies was measured using a PCR method (22) and quantitation of HKII mRNA showed a 66% decrease in NIDDM patients compared with healthy age- and weight-matched volunteers. To estimate the relative immunoreactive protein level of HKII we have raised an antipeptide antibody against the carboxy terminus of the human muscle HKII. In homogenate of muscle biopsies taken in the basal state the antibody recognized a dominant and specific band with an apparent molecular mass of ~ 100 kD, which corresponds to the cDNA-predicted molecular mass for human muscle HKII (102.4 kD). Densitometric scanning of autoradiograms showed a significant decrease (25%) in the relative level of HKII immunoreactive protein when compared with the control group. In parallel to the impaired HKII gene expression in muscle from NIDDM patients, we found significantly decreased muscle total GS activity and muscle GS mRNA level in the NIDDM group, thus confirming our previous results of decreased muscle GS mRNA in NIDDM patients obtained by a different methodology (17, 33). Moreover, significantly negative correlations were found between the gene expression and enzyme activity of HKII in the basal state (HKII mRNA and HKII activity) and fasting

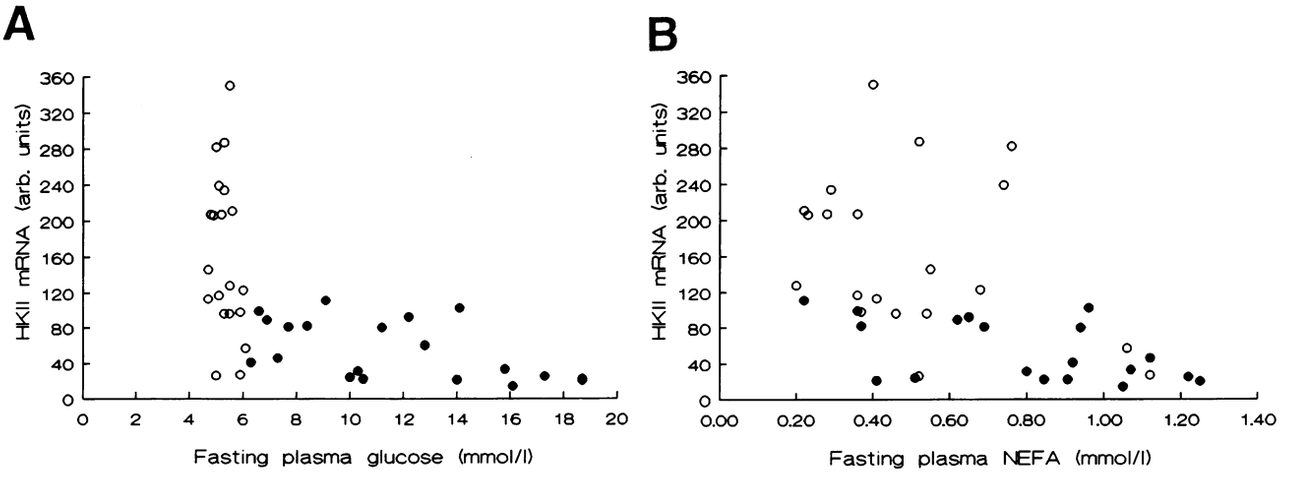

Figure 4. Muscle HKII mRNA levels were inversely correlated to both fasting plasma glucose (r = −0.73, P < 0.0001) (*A*) and plasma NEFA (r = −0.60, P < 0.0001) (*B*) in the entire group of study participants (n = 40). NIDDM: r = −0.54, P = 0.01 (glucose) and r = −0.40, P = 0.08 (NEFA); controls: r = −0.22, P = 0.34 (glucose), and r = −0.27, P = 0.26 (NEFA). *Filled circles,* NIDDM; *open circles,* control.

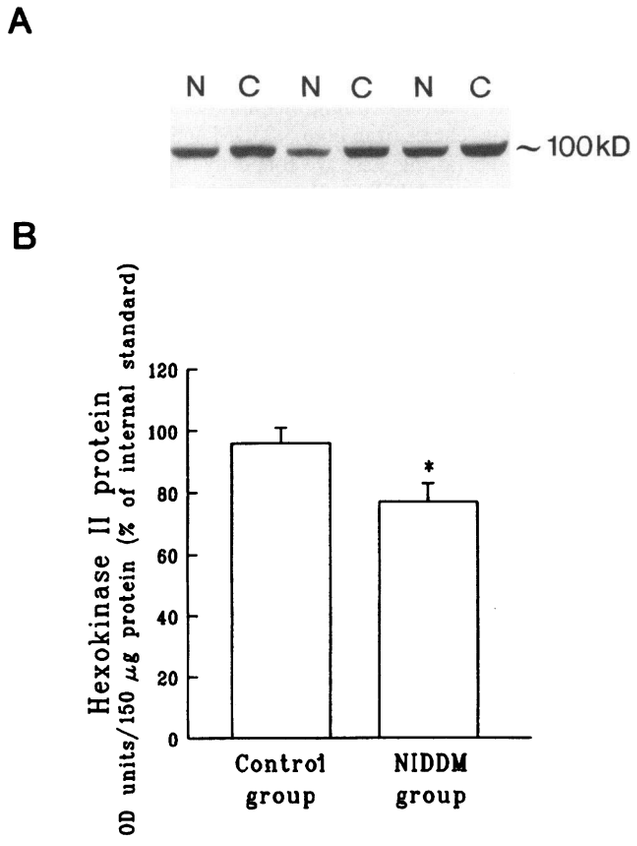

Figure 5. (*A*) Autoradiogram of immunoblotting analysis of HKII protein levels in skeletal muscle from NIDDM patients (*N*) and healthy control subjects (*C*) in the basal state. The blot is representative of 53 subjects (28 NIDDM patients and 25 control subjects; Table I). In all study participants a dominant band of ~ 100 kD was identified for HKII immunoreactive protein. (*B*) Results of densitometric scanning of autoradiograms. Muscle HKII protein in the basal state was significantly decreased in NIDDM patients (*P = 0.03). Values are mean±SE.

plasma concentrations of glucose and NEFA in the study participants. Thus, from these data it may seem that the impaired activity and gene expression of HKII are secondary to the diabetic state.

In favor of a secondary abnormality are data from a study of the KKA^{Y} NIDDM mouse model demonstrating significant reductions in muscle HKII mRNA, HKII protein, and HKII activity when compared with control mice and a restoring of the reduced HKII mRNA and protein levels to normal by the insulin-sensitizing drug pioglitazone (29). Similarly, both reduced muscle HKII mRNA and activity have been demonstrated in animal models of insulin-dependent diabetes mellitus and have been shown to be regulated by insulin (5, 34).

The finding of reduced levels of muscle HKII mRNA in NIDDM patients indicates an impaired transcription rate or an increased degradation of the transcript. A decreased transcription of the HKII gene might be caused by genetic variability in factors that regulate the level of HKII expression. We have recently examined the coding region of the HKII gene in NIDDM patients using single-strand conformational polymorphism analysis and nucleotide sequencing to determine whether these patients show genetic variability associated with or contributing to the disease (35). Four missense mutations were found (codons 142, 148, 497, and 844) of which the codon 142 variant was widespread with an allelic frequency of 19% among control subjects and 17% among NIDDM patients. However, no obvious relationships between whole-body insulin sensitivity or glucose effectiveness and the gene variant could be shown. Thus, no gene defects were identified in the coding region which were likely to be related to an increased HKII mRNA degradation.

To determine whether reduced muscle glucose transport and/or phosphorylation is a primary defect in the pathogenesis of NIDDM, whole-body glucose uptake has been measured during a hyperglycemic hyperinsulinemic clamp in diabetes-prone but glucose-tolerant offspring of NIDDM patients and muscle G6P concentration was assessed using ^{31}P NMR spectroscopy of gastrocnemius muscle (36). Both G6P concentration and whole-body insulin-stimulated glucose uptake were significantly lower in the offspring when compared with a matched control group, suggesting reduced glucose transport and/or glucose phosphorylation as a cause of reduced glucose uptake and that this defect might be an early event in the pathogenesis of NIDDM. Whether muscle HKII gene expression is impaired in glucose-tolerant offspring of NIDDM patients still has to be clarified, whereas muscle GLUT4 gene expression has been shown to be normal (37).

GS, which is the rate-determining enzyme in the muscle glycogen synthesis pathway, may be modified covalently by multiple phosphorylations of serine residues (38). Insulin activates GS covalently by reducing and increasing the activities of specific kinases and phosphatase, respectively (39, 40), whereas G6P allosterically activates the phosphorylated form of GS (38). The stimulatory effect of G6P at physiological concentrations on GS seems to be specific for GS and is most likely due to conformational changes of the enzyme which facilitates its dephosphorylation (41). Whether the physiologic consequences of the changes in muscle HKII gene expression in NIDDM patients have any influence on the GS activation in vivo is, however, uncertain. Two previous investigations have shown that when NIDDM patients and matched control subjects were examined during similar glucose utilization rates and similar circulating plasma insulin levels (isoglycemic hyperinsulinemic clamp conditions), diabetic patients had an increased intracellular concentration of free glucose and G6P in muscle tissue, indicating that the rate-limiting step in muscle glucose metabolism during isoglycemic hyperinsulinemia in patients with NIDDM may be located distal to the glucose phosphorylation step (13, 14). Also during fasting hyperglycemia NIDDM patients had increased levels of intracellular glucose and G6P, further indicating that the rate-limiting step in muscle glucose metabolism may be located distal to the glucose phosphorylation step (14). These data were further supported from studies of three groups of either metabolically normal, insulin-resistant nondiabetic, or overtly diabetic rhesus monkeys showing that under euglycemic hyperinsulinemic conditions significantly inverse relations exist between intracellular G6P and GS activation and insulin-stimulated glucose uptake, indicating a major defect distal to G6P in these insulin-resistant and diabetic monkeys (42). The insulin-resistant monkeys were also characterized by having increased levels of intracellular muscle G6P during euglycemic hyperinsulinemia. Whether the increase in intracellular glucose and G6P found in these studies may be extrapolated to the present protocol to suggest that glucose and its metabolite may have a downregulatory impact on HKII gene expression needs to be further investigated. Moreover, the finding that skeletal muscle glucose and G6P concentrations are

increased in NIDDM patients during either hyperglycemia or isoglycemic hyperinsulinemia does not exclude the possibility for decreased intracellular muscle glucose or G6P concentrations during euglycemia, which might point to an additional impairment in either glucose transport or phosphorylation. Data implying such defects have been published recently in a study using a novel triple tracer technique in obese diabetic and nondiabetic patients. During a euglycemic hyperinsulinemic clamp it was shown that transmembrane glucose transport and intracellular glucose phosphorylation in vivo are similarly impaired in skeletal muscle and may contribute to the insulin resistance found in these patients when compared with lean nondiabetic control subjects (12).

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