

Insulin-Induced Hexokinase II Expression Is Reduced in Obesity and NIDDM

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NIDDM and obesity are characterized by decreased insulin-stimulated glucose uptake in muscle. It has been suggested that impaired glucose phosphorylation to glucose-6-phosphate, catalyzed in muscle by hexokinase (HK)II, may contribute to this insulin resistance. Insulin is known to increase HKII mRNA, protein, and activity in lean nondiabetic individuals. The purpose of this study was to determine whether defects in insulin-stimulated HKII expression and activity could contribute to the insulin resistance of obesity and NIDDM. Fifteen lean nondiabetic control subjects, 17 obese nondiabetic subjects, and 14 obese NIDDM patients were studied. Percutaneous muscle biopsies of the vastus lateralis were performed in conjunction with leg balance and local indirect calorimetry measurements before and at the end of a 3-h euglycemic-hyperinsulinemic clamp (40 or 240 $\text{mU} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$). Leg glucose uptake in response to the 40-mU insulin infusion was higher in the lean control subjects ($2.53 \pm 0.35 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \text{ per } \cdot 100 \text{ ml leg vol}$) than in obese (1.46 ± 0.50) or NIDDM (0.53 ± 0.25 , $P < 0.05$) patients. In response to 240 mU insulin, leg glucose uptake was similar in all of the groups. In response to 40 mU insulin, HKII mRNA in lean control subjects was increased 1.48 ± 0.18 -fold ($P < 0.05$) but failed to increase significantly in the obese (1.12 ± 0.24) or NIDDM (1.14 ± 0.18) groups. In response to 240 mU insulin, HKII mRNA was increased in all groups (control subjects 1.48 ± 0.18 , $P < 0.05$ vs. basal, obese 1.30 ± 0.16 , $P < 0.05$, and NIDDM 1.25 ± 0.14 , $P < 0.05$). Under basal conditions, HKI and HKII activities did not differ significantly between groups. Neither the 40 mU nor the 240 mU insulin infusion affected HK activity. Total HKII activity was reduced in the obese subjects ($4.33 \pm 0.08 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \text{ muscle protein}$) relative to the lean control subjects (5.00 ± 0.08 , $P < 0.05$). There was a further reduction in the diabetic patients (3.10 ± 0.10 , $P < 0.01$ vs. the control subjects, $P < 0.01$ vs. the obese sub-

jects). Resistance to insulin's metabolic effects extends to its ability to induce HKII expression in obesity and NIDDM. *Diabetes* 47:387-394, 1998

NIDDM and obesity are characterized by decreased insulin-stimulated glucose uptake in muscle (1), the primary tissue responsible for disposal of a glucose load (2). Glycogen synthesis, catalyzed by glycogen synthase (GS), is quantitatively the most defective pathway, although glycolysis and glucose oxidation are also impaired (3). Potentially, defects in muscle glucose transport, glucose phosphorylation, and/or glycogen synthesis could account for the reduced rate of glucose disposal in diabetes and obesity (3-5). Abnormalities in the activation of GS (6-8) and glucose transport (9-11) by insulin consistently have been demonstrated in NIDDM. More recently, Bonadonna et al. (11) and Rothman et al. (12), using different approaches, have provided evidence that impaired glucose phosphorylation to glucose-6-phosphate (G-6-P), catalyzed in muscle by hexokinase (HK)II, may contribute to this abnormality. Rothman et al. showed that during hyperinsulinemic and hyperglycemic conditions, reduced muscle glucose uptake in NIDDM was associated with a lower muscle G-6-P content, as measured by ^{31}P nuclear magnetic resonance spectroscopy (12). Bonadonna et al., using an isotopic tracer technique with mathematical modeling (13), demonstrated defects in insulin-stimulated glucose transport and phosphorylation in patients with NIDDM (11). When muscle glucose transport was normalized by hyperglycemia, an independent defect in phosphorylation remained, indicating that the phosphorylation defect was not simply the result of diminished glucose transport with decreased supply of glucose to HKII. Taken together, these findings predict that HKII, which regulates glucose phosphorylation in muscle, may play a key role in the insulin resistance of NIDDM and obesity.

HKII expression is regulated by insulin in skeletal muscle. HKII activity is decreased in the muscle of streptozotocin-induced diabetic rats, and insulin completely reverses the defect (14). Using the euglycemic-hyperinsulinemic clamp technique, it has been found that insulin increases HKII mRNA and protein in rats (15). Insulin has also been shown to increase HKII mRNA at the level of transcription in L6 skeletal muscle cells (16). Most recently, we have shown that insulin infusion increases HKII mRNA, protein, and activity levels in vivo in percutaneous muscle biopsies in healthy control subjects (17). There is also evidence that HKII expression is impaired in NIDDM. Vestergaard et al. (18) have demonstrated that basal levels of HKII mRNA and pro-

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ANOVA, analysis of variance; FFA, free fatty acid; G-6-P, glucose-6-phosphate; GS, glycogen synthase; HK, hexokinase; MAP, mitogen-activated protein.

TABLE 1
Subject characteristics

	40 mU · m ⁻² · min ⁻¹ insulin			240 mU · m ⁻² · min ⁻¹ insulin		
	Control subjects	Obese subjects	NIDDM patients	Control subjects	Obese subjects	NIDDM patients
<i>n</i>	10	12	11	8	8	5
Age (years)	34 ± 3	34 ± 2	44 ± 3	37 ± 3	43 ± 4	38 ± 4
Sex (M/W)	7/3	5/7	5/6	8/0	6/2	2/3
Ethnicity (MA/A)	7/3	7/5	10/1	5/3	4/4	5/0
BMI (kg/m ²)	24 ± 1	30 ± 1*‡	33 ± 2*‡	25 ± 1	30 ± 1*‡	31 ± 1*‡
Body fat (%)	19 ± 1	32 ± 2*‡	31 ± 1*‡	20 ± 1	28 ± 2*‡	28 ± 4*‡
FPG (mmol/l)	5.1 ± 0.1	5.2 ± 0.1	11.4 ± 0.8*†‡§	5.5 ± 0.2	5.4 ± 0.2	9.3 ± 1.1*†‡§
HbA _{1c} (%)	—	—	9.0 ± 0.6	—	—	10.2 ± 0.6
Fasting insulin (μU/ml)	7 ± 1	12 ± 2	20 ± 2*†‡§	9 ± 2	10 ± 1	21 ± 3*†‡§

Data are means ± SE or *n*. **P* < 0.05 vs. control; †*P* < 0.05 vs. obese; ‡*P* < 0.05 vs. control 240; §*P* < 0.05 vs. obese 240. A, Anglo; FPG, fasting plasma glucose; MA, Mexican American.

tein are decreased in NIDDM patients compared with control subjects. HK activity in muscle has been reported to be either reduced (18) or normal to increased (19). However, little is known about the regulation of HKII expression by insulin in NIDDM or obesity.

The present investigation had three purposes: 1) to determine dose-response characteristics for the insulin-induced increase in HKII expression in lean nondiabetic subjects, 2) to establish whether insulin-stimulated HKII expression and activity are impaired in NIDDM and obesity, and 3) to determine whether decreased HKII activity is associated with decreased insulin-stimulated leg glucose uptake in NIDDM and obesity. Because of the well-known abnormality in insulin-mediated stimulation of muscle glucose storage and GS activity, these parameters were also examined in this study to put the HK results into context.

RESEARCH DESIGN AND METHODS

Subjects. A total of 54 volunteers participated in the study. These subjects composed three groups: lean control subjects (*n* = 18), obese nondiabetic subjects (*n* = 20), and NIDDM patients (*n* = 16). Clinical characteristics of the subjects are given in Table 1. The control and obese subjects had no significant medical problems and were not taking any medications. Normal glucose tolerance was confirmed by a 75-g oral glucose tolerance test performed within 1 month before the study (20). Diabetic patients had been diagnosed with the disease for an average of 3 years (range: 1 month to 7 years), had no other medical problems, and were not taking any medications except for insulin or an oral sulfonylurea agent. Two subjects received no medication for their diabetes; 11 were taking sulfonylureas; and 3 were taking insulin. Sulfonylureas were discontinued 48 h before the beginning of the study. Insulin (regular and NPH) was withheld for 24 h before the day of the study. Body composition was determined for all subjects using bioimpedance (RJL Bio-106 Spectrum Body Composition Analyzer, RJL System, Detroit, MI). Subjects were instructed to maintain their usual diet for at least 3 days and not to engage in heavy exercise for at least 2 days before the study.

The purpose, nature, and potential risks of the study were explained to all subjects, and informed written consent was obtained before their participation. The protocol was reviewed and approved by the institutional review board of The University of Texas Health Science Center at San Antonio.

Study design. All studies were conducted in the general clinical research center at the Audie L. Murphy Veterans Administration Hospital and began at 0730 after a 10-h overnight fast.

An arm vein was cannulated for infusion of insulin and glucose, and a hand vein was cannulated in a retrograde fashion and placed in a heated box (60°C) for sampling of arterialized blood. After a 60-min rest period, a percutaneous muscle biopsy of the vastus lateralis muscle was obtained as previously described (21). Muscle biopsy specimens, which ranged in weight from 75 to 200 mg, were immediately blotted free of blood, frozen in liquid nitrogen, and stored in nitrogen until assay. Immediately after the muscle biopsy, euglycemic-hyperinsulinemic clamps

were performed as previously described (22). Thirty-three of the subjects (*n* = 10 control subjects, *n* = 12 obese subjects, and *n* = 11 NIDDM patients) received an intravenous insulin (Humulin; Eli Lilly, Indianapolis, IN) infusion at a rate of 40 mU · min⁻¹ · m⁻² for 3 h. Twenty-one of the subjects (*n* = 8 control subjects, *n* = 8 obese subjects, and *n* = 5 NIDDM patients) received an insulin infusion of 240 mU · min⁻¹ · m⁻² for 3 h. Plasma arterial glucose was measured at 5- to 10-min intervals throughout the study, and a variable infusion of 20% glucose was infused to maintain euglycemia. After 3 h of insulin infusion, a second muscle biopsy was obtained at a site 4 cm distal to the first biopsy. During a 30-min basal period and during the final minutes of the insulin infusion, arterialized blood samples were obtained at 10-min intervals for determination of plasma insulin and free fatty acid (FFA) concentrations. After the second biopsy, the insulin infusion was discontinued and the study was concluded.

Most of the subjects (*n* = 40) underwent leg balance measurements in conjunction with the insulin clamps. In these subjects, a radial artery (20 gauge, Arrow International, Reading, PA), rather than a hand vein, was cannulated. A femoral vein (16 gauge, Arrow International) was also cannulated. During a 30-min period before each muscle biopsy, simultaneous sampling of radial arterial and femoral venous blood was performed to determine arteriovenous differences. Samples were obtained at 10-min intervals for glucose and lactate measurements. For leg gas exchange (leg indirect calorimetry), separate samples were obtained at 5-min intervals and immediately placed on ice. The arterial and venous samples were analyzed for O₂ and CO₂ content within 30 min. Immediately before each biopsy in these subjects, leg blood flow was determined in triplicate using mercury strain gauge plethysmography (Hokanson, Bellevue, WA).

Muscle biopsy processing. Muscle biopsies were divided into two portions. One portion was used for isolation of total RNA by a guanidinium isothiocyanate method (14). The other portion of the biopsy was processed for assay of GS and HKII and HKI activities (see below). Biopsies were homogenized using a Polytron (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*-aminobenzamide; 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 was removed and kept on ice, and the pellet was resuspended in the extraction buffer containing 0.1% Triton X-100. It has previously been shown that the supernatant and pellet represent cytosol and crude mitochondria, respectively, as determined by the marker enzymes GS and pyruvate dehydrogenase (21). Aliquots of the supernatant and solubilized pellet were kept on ice for assay of GS, HKI, and HKII activity.

Enzyme activity assays for HKII, HKI, and GS. We used the different temperature sensitivities of HKII and HKI to separate the activities of the two enzymes (23). Aliquots of cytosolic and crude mitochondrial fractions were either heated at 45°C for 1 h or kept on ice. Because HKII activity is inactivated at 45°C (23), HK activity assayed on the heated sample represents HKI activity, while activity assayed on the samples kept at 4°C represents total HK (HKI plus HKII) activity. HKII activity was determined as the difference between these values. HKII and HKI activities in the soluble and particulate fractions are consistent with the presence of HKII and HKI protein determined by immunoblot analysis using antibodies specific for the two isoforms (17). HK activity was determined using a modification (24) of an enzyme-linked fluorometric assay (23). The HK assay buffer consisted of 40 mmol/l Tris-HCl, pH 7.5; 100 mmol/l KCl; 20 mmol/l MgCl₂;

TABLE 2
Plasma arterial substrate and hormone concentrations

	Basal			Insulin		
	Control subjects	Obese subjects	NIDDM patients	Control subjects	Obese subjects	NIDDM patients
40 mU · m ⁻² · min ⁻¹ insulin						
Glucose (mmol/l)	5.3 ± 0.1	5.2 ± 0.1	9.6 ± 0.9*†	5.3 ± 0.2	5.1 ± 1.1	5.6 ± 0.2
Insulin (μU/ml)	7 ± 1	12 ± 2	20 ± 2*†	68 ± 3	73 ± 4	90 ± 4
FFA (mmol/l)	557 ± 50	617 ± 40	687 ± 54	74 ± 5	114 ± 15	216 ± 40‡§
240 mU · m ⁻² · min ⁻¹ insulin						
Glucose (mmol/l)	5.4 ± 0.2	5.4 ± 0.2	9.5 ± 0.9*†	5.4 ± 0.1	5.6 ± 0.2	5.5 ± 0.1
Insulin (μU/ml)	9 ± 2	10 ± 1	21 ± 3*†	618 ± 46	665 ± 24	696 ± 52‡
FFA (mmol/l)	610 ± 58	642 ± 23	601 ± 79	88 ± 10	118 ± 17	126 ± 26

Data are means ± SE. **P* < 0.05 vs. basal control; †*P* < 0.05 vs. basal obese; ‡*P* < 0.05 vs. insulin control; §*P* < 0.05 vs. insulin obese.

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, St. Louis, MO). Total assay volume was 1.0 ml, and the assay was started by adding 10 μl of muscle extract fractions (30–60 μg protein). The increase in fluorescence was monitored for 15 min using a fluorometer (Turner Model 112; Sequoia-Turner, Mountain View, CA) calibrated to 5 nmoles NADPH full scale. This assay was linear for at least 20 min, during which time <1% of substrates and cofactors were consumed.

RNase protection assays for HKII and HKI mRNAs. RNase protection assays for specific mRNAs were performed using Maxiscript and RPA kits (Ambion, Austin, TX). A fragment of human HKII cDNA (25) was used as a template to produce an antisense RNA probe that protected a product of 231 nt, and a fragment of human HKI cDNA (25) was used to produce a probe that protected a product of 396 nt. Two micrograms of total RNA from each biopsy were used in the protection assays. After RNase digestion, protected products were separated by urea-PAGE and quantified by PhosphorImage analysis (Molecular Dynamics, Sunnyvale, CA).

Analytical methods. Plasma glucose was measured with a Beckman glucose analyzer (Fullerton, CA). Plasma CO₂ content was calculated from measured plasma CO₂ tension and pH (IL 1420 BG3 Analyzer; Allied Instrumentation Laboratory, Lexington, MA) and adjusted to whole blood CO₂ content based on hemoglobin concentration, O₂ saturation, and blood pH (26). Blood O₂ content was measured with a co-oximeter (482 Co-oximeter; Allied Instrumentation Laboratory). Lactate concentrations were determined using whole blood deproteinization at the bedside with an equal volume of 7% perchlorate acid; supernatants were stored at -70°C until assay (27,28). Arterial plasma insulin was determined by radioimmunoassay, and plasma FFA was measured enzymatically (NEFA C kit; Wako Pure Chemical Industries, Osaka, Japan). Blood lactate concentration was determined by an enzymatic method (28).

Calculations. Whole body glucose uptake was calculated as the average glucose infusion rate during the 30-min period preceding the muscle biopsy, with a small correction for overfilling or underfilling the plasma glucose space (22). Whole blood glucose concentration was estimated from plasma glucose concentration and the hematocrit (29). Glucose, lactate, and CO₂ and O₂ balances across the leg were calculated as the product of blood flow and the radial artery minus femoral venous concentration difference and expressed in units of micromoles per minute per 100 ml leg tissue. Rates of leg carbohydrate oxidation were calculated using the equations of Frayn (30) modified for limb indirect calorimetry, including separate constants to correct for leg protein oxidation under basal conditions

and during moderate hyperinsulinemia (31). Leg net glucose storage was calculated as the difference between leg glucose uptake and the sum of leg glucose oxidation and net balance of lactate, expressed in glucose equivalents.

Statistical methods. Subject characteristics, substrate and hormone levels, blood flow measurements, glucose metabolism values, and enzyme activities are presented as means ± SE. Differences between the three groups were tested by analysis of variance (ANOVA). When an analysis showed a significant (*P* < 0.05) difference between the group means, Fisher's PLSD test was performed to compare individual means. HKI and HKII mRNA levels are presented as fold stimulation over the basal values. The ability of insulin to increase fold stimulation of these parameters, relative to basal values (no fold stimulation = 1), was evaluated using one-tailed paired Student's *t* tests.

RESULTS

Substrate and insulin concentrations. Plasma arterial concentrations of substrates and insulin are given in Table 2. Basal glucose concentrations did not differ between the control and obese subjects and were maintained at the basal level during hyperinsulinemia. Basal glucose concentrations were significantly higher in the diabetic patients and decreased during hyperinsulinemia to a level similar to that of the control subjects. Basal insulin concentrations were higher in the diabetic patients than in the obese and control subjects. At each level of experimental hyperinsulinemia, steady-state plasma insulin concentrations were similar in all three groups. Plasma FFA levels were suppressed by both levels of hyperinsulinemia, but during the 40 mU insulin infusion, FFA levels in the obese and control subjects were suppressed to a greater degree than in the diabetic patients.

Whole body glucose metabolism. The glucose infusion rates required to maintain euglycemia during the last 30 min of each insulin infusion are given in Table 3. Obese nondiabetic subjects and patients with NIDDM had glucose infusion

TABLE 3
Glucose infusion rates required to maintain euglycemia

	40 mU · m ⁻² · min ⁻¹ insulin			240 mU · m ⁻² · min ⁻¹ insulin		
	Control subjects	Obese subjects	NIDDM patients	Control subjects	Obese subjects	NIDDM patients
<i>n</i>	10	12	11	8	8	5
Glucose infusion (mg/min kg)	8.3 ± 0.9	3.3 ± 0.3*	1.9 ± 0.2*	11.2 ± 1.3*	6.5 ± 0.5†	6.1 ± 1.1†

**P* < 0.05 vs. control (40 mU · m⁻² · min⁻¹); †*P* < 0.05 vs. control (240 mU · m⁻² · min⁻¹).

TABLE 4
Leg blood flow

	40 mU · m ⁻² · min ⁻¹ insulin			240 mU · m ⁻² · min ⁻¹ insulin		
	Control subjects	Obese subjects	NIDDM patients	Control subjects	Obese subjects	NIDDM patients
Basal	1.9 ± 0.2	2.5 ± 0.3	2.2 ± 0.3	2.1 ± 0.3	2.5 ± 0.3	2.1 ± 0.3
Insulin	2.9 ± 0.3*	3.4 ± 0.8	4.4 ± 0.9*	3.4 ± 0.9*	4.4 ± 0.9*	3.4 ± 0.3*

Data are means ± SE and are given in milliliters per minute per 100 milliliters leg tissue. **P* < 0.05 vs. basal.

rates during the 40 mU · m⁻² · min⁻¹ insulin infusion that were reduced to ~40 and 23% of values in lean control subjects (*P* < 0.05). During the 240 mU · m⁻² · min⁻¹ insulin infusion, glucose infusion rates in these groups were 58 and 54%, respectively, of values in lean control subjects (*P* < 0.05).

Leg blood flow. Rates of leg blood flow are given in Table 4. Basal values were similar in all three groups, and blood flow increased in all groups during both levels of hyperinsulinemia. There were no significant differences among the groups during either the 40 or the 240 mU · m⁻² · min⁻¹ insulin infusions.

Leg muscle glucose metabolism. In the three groups overall, both the 40 and 240 mU insulin infusions significantly increased leg glucose uptake, glucose oxidation, nonoxidative glycolysis, and glucose storage (*P* < 0.05) (Table 5). During the 40 mU infusion, each of these parameters was highest in the control subjects, followed by the obese subjects, and then the patients with NIDDM. During the 240-mU infusion, these parameters were similar in the control and obese subjects but tended to be lower in the NIDDM patients.

HK mRNAs. The fold stimulation above baseline of HKII and HKI mRNA is shown in Fig. 1. A total of 3 h of physiological hyperinsulinemia increased HKII mRNA in the control group by 1.48 ± 0.18-fold (*P* < 0.05) over basal values. In contrast, there was no significant stimulation of HKII mRNA in the obese (1.14 ± 0.23) or diabetic (1.14 ± 0.18) groups with this level of hyperinsulinemia. During the 240 mU insulin infusion, HKII mRNA in the control group was stimulated to the same degree (1.45 ± 0.18-fold, *P* < 0.05 vs. basal) as during the 40

mU insulin infusion. With this supraphysiological dose of insulin, the average fold stimulation of HKII mRNA in the obese (1.30 ± 0.16, *P* < 0.05) and diabetic (1.25 ± 0.14, *P* < 0.05) groups increased significantly above baseline but remained less than in the control subjects. There was no stimulation of HKI mRNA in response to insulin. When data from all three groups for both insulin infusions were pooled, there was a weak correlation between the insulin-stimulated increase in HKII (*r* = 0.27, *P* = 0.09) and HKI (*r* = 0.30, *P* = 0.06) mRNA and insulin-stimulated leg glucose uptake, although neither correlation coefficient was statistically significant.

HK and GS activities. Both HKI and HKII activities were present in the muscle biopsy specimens (Tables 6 and 7). HKI activity was confined primarily to the soluble fraction of the muscle extracts, while HKII activity was apportioned between the soluble and particulate fractions. Both in basal conditions and during insulin infusion, more HKII activity was associated with the particulate than with the soluble fraction of the muscle extract (*P* < 0.05).

Neither the 40 nor the 240 mU · m⁻² · min⁻¹ infusion of insulin for 3 h increased HKII or HKI activity in the control subjects, the obese subjects, or the NIDDM patients. Two-way ANOVA, however, revealed that regardless of condition (basal or insulin), HKII activity in the soluble and particulate fractions was greater in the nondiabetic control subjects and obese subjects than in the patients with NIDDM (*P* < 0.01 for both). Total HKII activity (soluble plus particulate) was greatest in the lean nondiabetic control subjects, intermediate in

TABLE 5
Effects of insulin on leg glucose metabolism

	Basal			Insulin		
	Control subjects	Obese subjects	NIDDM patients	Control subjects	Obese subjects	NIDDM patients
40 mU · m ⁻² · min ⁻¹						
<i>n</i>	8	6	5	8	6	5
Glucose uptake	0.20 ± 0.07	0.13 ± 0.07	0.05 ± 0.04	2.53 ± 0.35	1.46 ± 0.50	0.53 ± 0.25*
Glucose oxidation	0.25 ± 0.06	0.37 ± 0.16	0.41 ± 0.13	0.90 ± 0.11	0.71 ± 0.27	0.34 ± 0.13
Nonoxidative glycolysis	0.06 ± 0.02	0.01 ± 0.01	0.04 ± 0.01	0.15 ± 0.04	0.03 ± 0.07	0.08 ± 0.01
Glucose storage	-0.12 ± 0.07	-0.26 ± 0.18	-0.40 ± 0.13	1.48 ± 0.32	0.72 ± 0.45	0.11 ± 0.32*
240 mU · m ⁻² · min ⁻¹						
<i>n</i>	6	9	6	6	9	6
Glucose uptake	0.04 ± 0.04	0.26 ± 0.06*	0.14 ± 0.05	3.96 ± 0.87	4.45 ± 0.99	2.8 ± 0.68
Glucose oxidation	0.24 ± 0.11	0.49 ± 0.14	0.34 ± 0.10	1.01 ± 0.29	1.39 ± 0.13	0.75 ± 0.29
Nonoxidative glycolysis	0.12 ± 0.05	-0.01 ± 0.03	0.06 ± 0.04	0.41 ± 0.14	0.24 ± 0.14	0.27 ± 0.22
Glucose storage	-0.30 ± 0.13	-0.22 ± 0.16	-0.26 ± 0.12	2.54 ± 0.79	2.81 ± 0.82	1.78 ± 0.59

Data are means ± SE and are given in micromoles per minute per 100 milliliters leg volume. **P* < 0.05 vs. control.

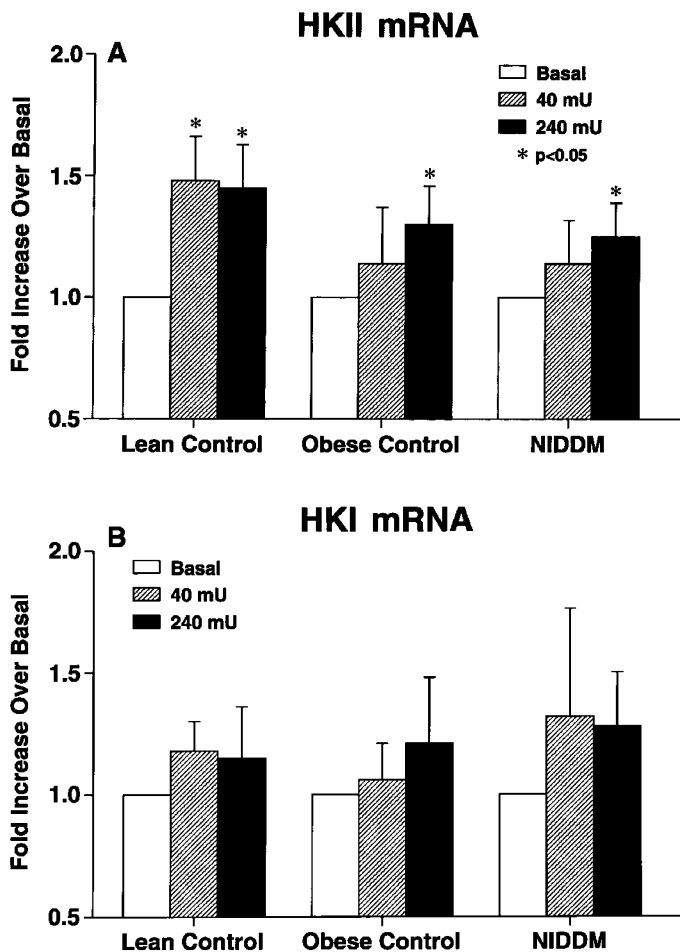


FIG. 1. Effect of insulin infusion on HKII (A) and HKI (B) mRNA in muscle biopsies from lean nondiabetic control subjects, obese nondiabetic control subjects, and patients with NIDDM. Basal values were set to 1.0, and data during the insulin infusion are expressed as fold stimulation (mean \pm SE).

the obese nondiabetic subjects ($P < 0.05$ vs. lean control subjects), and least in the patients with NIDDM ($P < 0.01$ vs. obese control subjects). A similar analysis showed that total HKI activity was reduced significantly in the diabetic patients (1.45 ± 0.05 pmol \cdot min $^{-1}$ per μ g muscle protein) relative to both obese subjects (2.34 ± 0.04 , $P < 0.01$) and lean control subjects (2.13 ± 0.10 $P < 0.04$, $P < 0.01$).

Two-way ANOVA revealed that, overall, insulin significantly increased GS activity in all groups ($P < 0.01$) (Table 8). Basal GS activity measured in the presence of 0.1 mmol/l G-6-P was similar in the control (0.49 ± 0.16 nmol \cdot min $^{-1}$ per μ g muscle protein) and obese (0.53 ± 0.10) subjects but was \sim 40% lower in the patients with NIDDM (0.29 ± 0.09 , $P < 0.05$ vs. control, $P < 0.01$ vs. obese). Basal GS activity assayed in the presence of 10 mmol/l G-6-P was higher in the lean (4.45 ± 0.79) than in the obese (3.67 ± 0.39 , $P < 0.01$) control subjects. It was even further reduced in the diabetic patients (2.47 ± 0.42 , $P < 0.01$ vs. control and obese). Basal GS fractional velocity was similar in the three groups. It approximately doubled in response to insulin in the control and obese groups ($P < 0.01$) but only increased by 35% in the diabetic patients ($P < 0.05$). When insulin-stimulated results from all three groups were pooled, there was a significant correlation between GS activity measured at 0.1 mmol/l G-6-P and insulin-stimulated leg muscle glucose storage ($r = 0.51$, $P = 0.02$).

DISCUSSION

Resistance to insulin's ability to promote muscle glucose uptake is a key feature of NIDDM and obesity. Glycogen synthesis accounts for the majority of glucose metabolism in skeletal muscle during hyperinsulinemia (6,7,32,33), and abnormalities in the regulation of GS activity are thought to contribute to the insulin resistance in patients with NIDDM and obesity (6,7,8,34,35). Defects in glycogen synthesis, however, could result from a more proximal abnormality, i.e., impaired glucose transport and/or phosphorylation. A more proximal abnormality could limit the ability of muscle cells to store glycogen, independent of the impairment in GS activity. Decreased glucose transport has consistently been

TABLE 6
HKII activities

	Control subjects	Obese subjects	NIDDM patients
<i>n</i>	10	12	11
Basal/40 mU			
Soluble	1.99 \pm 0.35/1.73 \pm 0.37	1.38 \pm 0.22/1.16 \pm 0.25	0.97 \pm 0.13*/1.34 \pm 0.24
Particulate	2.96 \pm 0.28/3.32 \pm 0.50	2.59 \pm 0.37/2.61 \pm 0.40	2.16 \pm 0.26/2.44 \pm 0.29
<i>n</i>	8	8	5
Basal/240 mU			
Soluble	1.60 \pm 0.20/1.50 \pm 0.17	1.63 \pm 1.41/1.70 \pm 0.43	1.16 \pm 0.51/0.73 \pm 0.27
Particulate	3.23 \pm 0.72/3.59 \pm 0.99	2.86 \pm 0.47/3.40 \pm 0.67	1.95 \pm 0.93/1.67 \pm 0.50
Pooled means			
<i>n</i>	18	20	16
Soluble	1.70 \pm 0.03	1.47 \pm 0.03	1.05 \pm 0.04†‡
Particulate	3.29 \pm 0.06	2.86 \pm 0.06	2.05 \pm 0.07†‡
Total	5.00 \pm 0.08	4.33 \pm 0.66*	3.10 \pm 0.10†‡

Data are means \pm SE and are given in picomoles per minute per microgram extract protein. Because there were no significant differences between basal- and insulin-stimulated activities, these values were pooled, and these marginal means were analyzed by ANOVA. * $P < 0.05$ vs. control; † $P < 0.01$ vs. control; ‡ $P < 0.01$ vs. obese.

TABLE 7
HKI activities

	Control subjects	Obese subjects	NIDDM patients
<i>n</i>	10	12	11
Basal/40 mU			
Soluble	1.76 ± 0.29/1.35 ± 0.26	1.53 ± 0.22/1.17 ± 0.14	1.03 ± 0.21/1.23 ± 0.19
Particulate	0.61 ± 0.14/0.49 ± 0.16	0.40 ± 0.06/0.46 ± 0.06	0.39 ± 0.05/0.4 ± 0.06
<i>n</i>	8	7	5
Basal/40 mU			
Soluble	1.58 ± 0.33/1.43 ± 0.28	1.85 ± 0.46/2.80 ± 0.76	0.92 ± 0.23/0.76 ± 0.18
Particulate	0.65 ± 0.14/0.65 ± 0.20	0.62 ± 0.17/0.54 ± 0.12	0.44 ± 0.22/0.61 ± 0.37
<i>n</i>	18	19	16
Pooled means			
Soluble	1.53 ± 0.04	1.84 ± 0.03	0.99 ± 0.04*†
Particulate	0.60 ± 0.02	0.50 ± 0.02	0.46 ± 0.02
Total	2.13 ± 0.04	2.34 ± 0.04	1.45 ± 0.05*†

Data are means ± SE and are given in picomoles per minute per microgram extract protein. Because there were no significant differences between basal- and insulin-stimulated activities, these values were pooled, and these marginal means were analyzed by ANOVA. **P* < 0.01 vs. control; †*P* < 0.01 vs. obese.

shown in experimental models of diabetes (36–40) and both in vitro (9,41–43) and in vivo in diabetic (10,11) humans. Further evidence for decreased insulin-stimulated transport and/or phosphorylation in NIDDM has been provided by Rothman et al. (12). They showed that during hyperglycemia and hyperinsulinemia, reduced muscle glucose uptake was associated with a lower muscle G-6-P content (measured by ³¹P nuclear magnetic resonance spectroscopy) as compared with control subjects. More recently, Bonadonna et al. used an isotopic tracer technique with mathematical modeling (13) to demonstrate defects in both glucose transport and phosphorylation in patients with NIDDM (11). The defect in glucose phosphorylation (~80%) was greater than the defect in transport (~40%). When muscle glucose transport in

NIDDM was normalized by experimental hyperglycemia, the defect in phosphorylation persisted, indicating that the impairment in muscle glucose phosphorylation was not simply due to a decreased supply of glucose to HK (11). Thus, a growing body of evidence suggests that impaired glucose phosphorylation may be the rate-determining defect responsible for decreased insulin-mediated muscle glucose uptake in NIDDM and obesity. The recent finding that a 4-h insulin infusion significantly increases HKII mRNA, protein, and activity levels in healthy control subjects (17) raised the possibility that HKII expression is an important site of insulin resistance in NIDDM and obesity.

The purpose of the present study was to determine whether insulin-stimulation of HK expression is impaired in

TABLE 8
Glycogen synthase activities

	Control subjects	Obese subjects	NIDDM patients
<i>n</i>	10	12	11
Basal/40 mU			
0.1 mmol/l G-6-P	0.51 ± 0.14/0.96 ± 0.19	0.55 ± 0.10/0.80 ± 0.14	0.32 ± 0.07/0.54 ± 0.14
10 mmol/l G-6-P	4.22 ± 0.59/4.24 ± 0.39	3.59 ± 0.42/3.11 ± 0.39	2.22 ± 0.21/2.53 ± 0.53
Fractional velocity	0.11 ± 0.02/0.22 ± 0.04	0.16 ± 0.03/0.29 ± 0.06	0.16 ± 0.05/0.21 ± 0.05
<i>n</i>	8	8	5
Basal/40 mU			
Soluble	0.47 ± 0.18/1.21 ± 0.26	0.51 ± 0.12/1.20 ± 0.30	0.23 ± 0.13/0.40 ± 0.19
Particulate	474 ± 1.04/4.89 ± 0.85	3.78 ± 0.34/3.72 ± 0.54	3.01 ± 0.88/2.56 ± 0.88
Fractional velocity	0.09 ± 0.02/0.23 ± 0.03	0.13 ± 0.03/0.31 ± 0.06	0.08 ± 0.02/0.15 ± 0.04
<i>n</i>	18	20	16
Pooled means, basal/insulin			
Soluble	0.49 ± 0.16/1.07 ± 0.22*	0.53 ± 0.10/0.96 ± 0.20*	0.29 ± 0.09†‡/0.50 ± 0.16*
Particulate	4.45 ± 0.79/4.53 ± 0.59	3.67 ± 0.39†/3.35 ± 0.45†	2.47 ± 0.42†‡/2.54 ± 0.64†‡
Fractional velocity	0.10 ± 0.02/0.22 ± 0.03*	0.15 ± 0.03/0.30 ± 0.06*†	0.14 ± 0.04/0.19 ± 0.04*

Data are means ± SE and are given in picomoles per minute per microgram extract protein. Because there were no significant differences between basal- and insulin-stimulated activities, these values were pooled, and these marginal means were analyzed by ANOVA. **P* < 0.01 vs. control; †*P* < 0.01 vs. obese.

NIDDM and obesity. A total of 3 h of physiological hyperinsulinemia increased HKII mRNA levels by ~50% in lean control subjects but had no effect in NIDDM and obese individuals. After exposure to supraphysiological doses of insulin, there was no further stimulation of HKII mRNA in lean control subjects. In contrast, during the supraphysiologic ($240 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) insulin infusion, muscle HKII mRNA levels in NIDDM patients and obese subjects increased significantly above baseline, although they did not return to the level observed in lean control subjects. Thus, resistance to insulin's metabolic effects extends to its ability to induce HKII expression in NIDDM and obesity.

Using wortmannin to inhibit PI 3-kinase activity and a mitogen-activated protein (MAP) kinase (MAP kinase/ER kinase kinase) inhibitor to ablate MAP kinase activity, it has been shown that in L6 myocytes, insulin induction of HKII expression is mediated by insulin activation of PI 3'-kinase alone (44). Inhibition of insulin stimulation of the MAP kinase pathway did not suppress insulin stimulation of HKII expression (44). The metabolic effects of insulin, such as its ability to increase glycogen synthesis or promote GLUT4 translocation, are thought to occur through activation of PI 3'-kinase (45–47), although some role for the MAP kinase pathway has not been ruled out completely (48). Therefore, our observation that the resistance to insulin's metabolic effects parallels the resistance to insulin's stimulatory effect on HKII expression lends credence to the notion that the defect in insulin signaling in NIDDM resides along the PI 3'-kinase pathway.

The present study design allowed us to determine whether decreased insulin-stimulated muscle glucose uptake in obesity and NIDDM was associated with a concomitant decrease in HK activity. Total HKII activity was significantly higher in the control subjects ($5.0 \pm 0.08 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$), followed by the obese subjects (4.33 ± 0.66), and then the diabetic patients (3.10 ± 0.10). These results are in agreement with those of Vestergaard et al. (18), who found reduced basal HKII mRNA, protein, and activity in patients with NIDDM. However, another study found that there were no differences in total HK activity in whole extracts of muscle biopsies taken from patients with NIDDM or their control subjects (19). The reasons for this discrepancy are not apparent, but in that study (19), no attempt was made to separate HKI and HKII activities. In addition, the sample size in the present study was greater than either of the two previous studies. Differences in assay methods, such as separation of HKI and HKII activities or differentiation of soluble and particulate fractions in the present study, may also account for the differences in the results.

Obese nondiabetic subjects had HKII activity and a response of HKII mRNA to insulin that was intermediate between lean nondiabetic control subjects and obese patients with NIDDM. This suggests that diabetes confers additional insulin resistance with respect to HKII expression over that of obesity alone. In the present study, this was also true for the other facets of insulin action, including leg glucose uptake, storage, and GS activity.

Because HKI mRNA is not increased by insulin infusion, it might be predicted that insulin-resistant subjects would have normal HKI activity. In keeping with this notion, obese and lean nondiabetic subjects had equivalent HKI activities. In contrast, there was a slight decrease in total HKI activity in

patients with NIDDM. This decrease represents only a small portion of all HK activity present in the muscle. The reason for this is unclear.

In summary, the present findings show that physiological hyperinsulinemia maximally stimulates HKII mRNA in healthy nondiabetic subjects. In obese subjects and in patients with NIDDM, physiological hyperinsulinemia did not increase HKII mRNA. These observations extend the insulin resistance from metabolism to gene expression in NIDDM and obesity. However, pharmacological insulin concentrations were able to overcome, in part, the insulin resistance for HKII mRNA in obese and NIDDM patients.

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