Regulation of Skeletal Muscle Hexokinase II by Insulin in Nondiabetic and NIDDM Subjects

Yolanta T. Kruszynska, Mim I. Mulford, Joseph Baloga, Joseph G. Yu, and Jerrold M. Olefsky

Impaired muscle glucose phosphorylation to glucose-6-phosphate by hexokinases (HKs)-I and -II may contribute to insulin resistance in NIDDM and obesity. HK-II expression is regulated by insulin. We tested the hypothesis that basal and insulin-stimulated expression of HK-II is decreased in NIDDM and obese subjects. Skeletal muscle HK-I and HK-II activities were measured in seven lean and six obese normal subjects and eight patients with NIDDM before and at 3 and 5 h of a hyperinsulinemic (80 mU · m^{-2} · min^{-1}) euglycemic clamp. To assess whether changes in HK-II expression seen during a glucose clamp are likely to be physiologically relevant, we also measured HK-I and HK-II activity in 10 lean normal subjects before and after a high-carbohydrate meal. After an overnight fast, total HK, HK-I, and HK-II activities were similar in lean and obese control subjects; but HK-II was lower in NIDDM patients than in lean subjects (1.42 ± 0.16 [SE] vs. 2.33 ± 0.24 nmol · min^{-1} · mg^{-1} molecular weight, $P < 0.05$) and accounted for a lower proportion of total HK (33 ± 3 vs. 47 ± 3%, $P < 0.025$). HK-II (but not HK-I) activity increased during the clamp in lean and obese subjects by 34 and 36% after 3 h and by 14 and 22% after 5 h of hyperinsulinemia; no increase was found in the NIDDM patients. In the lean subjects, muscle HK-II activity also increased by 15% 4 h after the meal, from 2.47 ± 0.19 basally to 2.86 ± 0.28 nmol · min^{-1} · mg^{-1} protein ($P < 0.05$). During the clamps, muscle HK-II activity correlated with muscle citrate synthase activity in the normal subjects ($r = 0.58$, $P < 0.05$) but not in the NIDDM patients. A weak relationship was noted between muscle HK-II activity and glucose disposal rate at the end of the clamp when all three groups were combined ($r = 0.49$, $P < 0.05$). In summary, NIDDM patients have lower muscle HK-II activity basally and do not increase the activity of this enzyme in response to a 5-h insulin stimulus. This defect may contribute to their insulin resistance. In nondiabetic obese subjects, muscle HK-II expression and its regulation by insulin are normal.
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In NIDDM and other insulin-resistant groups, the defect in insulin-stimulated glucose disposal correlates with impaired glucose storage as glycogen in muscle (1–4). To be stored as muscle glycogen, glucose must first be transported into muscle by the insulin-regulated glucose transporter (GLUT4) (1,5). It must then be phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase (HK). This step is crucial for efficient glucose uptake, because it maintains the transmembrane glucose gradient for facilitated diffusion of glucose.

Two isozymes of HK (HK-I and HK-II) are present in skeletal and cardiac muscle. In rodent skeletal muscle, HK-II predominates, accounting for >90% of the total (6–10). Human muscle contains a higher proportion of HK-I, HK-II accounting for 40–70% of the total (11–13). HK-II is of interest in relation to regulation of glucose uptake, since it is expressed essentially in insulin-sensitive tissues (skeletal muscle, heart, and adipose tissue), and HK-II gene transcription is regulated acutely by insulin (9,10,14) and possibly glucose itself (15). HK-II expression is reduced in muscle from diabetic rats and mice (6,16), implying an important role for insulin in the maintenance of basal levels of this isozyme in muscle. Muscle HK-II mRNA increases several-fold when insulin is infused for 3–6 h in humans (14) and rodents (9), and there is a corresponding increase in immunodetectable enzyme and HK-II activity in muscle homogenates. Decreased activity of HK-II in muscle from NIDDM patients has recently been reported (12). This study did not, however, examine the relationship with insulin sensitivity or abnormal muscle glucose handling, nor the ability of insulin to increase the synthesis of this enzyme in NIDDM.

Our aim was to test the hypothesis that basal and insulin-stimulated expression of HK-II is decreased in insulin-resistant NIDDM patients and obese subjects and to relate the activity of this enzyme to whole body insulin sensitivity. To determine whether abnormalities in HK-II regulation seen during a glucose clamp may be physiologically relevant, we also measured HK-II activity in muscle of lean subjects before and after a high-carbohydrate meal.

RESEARCH DESIGN AND METHODS

Muscle enzyme activities were determined in vastus lateralis muscle biopsies obtained before and during a 5-h hyperinsulinemic euglycemic clamp from 8 patients with NIDDM (M:W ratio = 6:2, BMI 32 ± 3.3 [SD] kg/m^2, age 48.4 ± 11.3 years) and 13 normal control subjects (7 lean: M:W ratio = 6:1, BMI 24.3 ± 2.6 kg/m^2, age 38.3 ± 7.2 years; 6 obese: M:W ratio = 5:1, BMI 35.5 ± 3.3 kg/m^2, age 43.3 ± 7.9 years). The diabetic group included one African-American and two Mexican-American subjects. The remaining diabetic subjects and all the control subjects were of European ancestry. Muscle enzyme activities were also studied in 10 lean control men (41.5 ± 5.8 years of age, BMI 24.8 ± 4.3 kg/m^2) before and after a high-

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ANOVA, analysis of variance; CV, coefficient of variation; G-6-P, glucose-6-phosphate; HGO, hepatic glucose output; HK, hexokinase; R_a, rate of glucose appearance; R_d, rate of glucose disappearance.

carbohydrate meal. Two of the subjects in this group were African-American, and eight were of European ancestry. Normal subjects had a normal 75-g oral glucose tolerance test (17). The diabetic patients were asked to continue with their usual diet but to stop their oral hypoglycemic agents for 2 weeks before study. HbA_{1c} in the diabetic patients was 9.0 ± 2.5%; in the 13 normal subjects participating in the glucose clamp study, it was 5.1 ± 0.7%. The study was approved by the human subjects internal review board of the University of California, San Diego. Written informed consent was obtained from each subject.

Hyperinsulinemic euglycemic clamps. Studies were performed in the morning after a 10- to 12-h overnight fast. At 0400, an 18-gauge cannula was inserted into an antecubital vein, and a constant infusion of [3-^{3}H]glucose (0.25 µCi/min) (New England Nuclear, Boston, MA) was started. For blood sampling, a cannula was inserted retrograde into a distal forearm vein, the hand being kept in a hand warmer at 70°C. After each blood sample, this cannula was flushed with 0.15 mol/l NaCl in water. After four basal blood samples were taken for measurement of plasma glucose concentration and specific activity, insulin, and C-peptide, an intravenous infusion of insulin (Humulin S, Eli Lilly, Indianapolis, IN), diluted in 0.15 mol/l saline containing 1% wt/vol human albumin, was begun at 80 mU · m^{-2} · min^{-1} from a Harvard syringe pump. Potassium and phosphate were given intravenously to maintain normal serum levels of these ions. Blood glucose was measured every 5 min, and the blood glucose concentration was clamped at 4.4 mmol/l for 5 h by adjusting the rate of infusion of a solution of 20% (wt/vol) glucose in water (18). Blood samples for glucose concentration and specific activity were taken every 15 min until 270 min and then every 10 min until 300 min.

A muscle biopsy (200–300 mg) was obtained from the vastus lateralis under local lignocaine anesthesia (19), before the basal blood samples and again at 180 and 300 min of the glucose clamp. The clamp was prolonged beyond 300 min until completion of the last muscle biopsy. Tissue was blotted free of blood and immediately frozen in liquid nitrogen, under which it was stored until used for measurement of enzyme activities.

Whole body glucose and lipid oxidation. Substrate oxidation rates in the basal state and during the glucose clamps were determined by indirect calorimetry, as previously described (20). O_2 consumption and CO_2 production were measured for 15 min during the second half of each 30 min of the study, and the means of the values during the last 10 min of the measurement interval were used for calculations. A timed (~5-h) basal urine sample was obtained, and a further sample was obtained at the end of the study for determination of urinary nitrogen (N) excretion rates during the basal and clamp periods. Carbohydrate and lipid oxidation was calculated from standard equations (21,22). In some subjects, negative lipid oxidation rates were observed during the glucose clamp. These negative values are thought to be numerically equivalent to the net amount of lipid synthesized (21,23). As discussed previously (20), when negative rates of lipid oxidation were obtained, glucose oxidation was calculated as 1.34(1.00 VCO_2 – 4.88 N) to correct for glucose that is converted into fat (21,23). Nonoxidative glucose disposal during the glucose clamp was calculated by subtracting the amount of glucose oxidized and that excreted in the urine from total plasma glucose disappearance rate (R_d).

Muscle enzyme activities in response to a high-carbohydrate meal. After a 10- to 12-h overnight fast, an 18-gauge cannula was inserted into an antecubital vein of 10 lean normal subjects for blood sampling. After two basal blood samples, subjects consumed a standard meal (1,040 calories, 64% carbohydrate, 25% fat, and 11% protein). Blood samples were obtained at 30, 60, 90, 120, 150, 180, and 240 min for measurement of plasma glucose and insulin concentrations. A muscle biopsy (200 mg) was obtained before the basal blood samples and at 4 h after the start of the meal.

Muscle HK and citrate synthase activities. Samples of muscle (~50–100 mg) were homogenized (Polytron Kinematica, Lucerne, Switzerland) at 4°C in 900 µl of 20 mmol/l Tris/HCl buffer (pH 8.1), containing 5 mmol/l EDTA, 250 mmol/l sucrose, 5 mmol/l glucose, and 5 mmol/l $MgCl_2$. After centrifugation of the homogenate (3,500 rpm for 10 min at 4°C), the supernatant was sonicated (Misonix, Farmingdale, NY) and freeze-thawed twice to disrupt mitochondria. Samples were then centrifuged at 14,000*g* for 2 min at 4°C, and the supernatant was used for spectrophotometric assays of HK and citrate synthase activity at 22°C. Citrate synthase activity was measured by using oxaloacetate and acetyl CoA as substrates and the dye, 5′-dithiobis-2-nitrobenzoic acid (DTNB) (24). The linear slope of the absorbance change with time (3–6 min) at 412 nm was used for calculation of activity.

HK was measured by coupling G-6-P production to NADPH formation via the G-6-P dehydrogenase reaction. One aliquot of the 14,000*g* supernatant was assayed to determine total HK activity (HK-I and HK-II), and another aliquot was heated for 1 h at 45°C and then assayed for heat-stable primarily HK-I activity (25). HK-II activity was determined by subtraction of HK-I from total HK activity. Reaction mixtures (1.06 ml) included the following: 40 µl of the 14,000*g* tissue extract and 1.02 ml of assay mix (50 mmol/l Tris, 5 mmol/l $MgCl_2$, 5 mmol/l glucose, 0.02% wt/vol bovine serum albumin, 2.5 mmol/l dithiothreitol, 0.5 mmol/l NADP, 1 U/ml G-6-P dehydrogenase [Boehringer Mannheim, Mannheim, Germany] and 2 mmol/l ATP, which was added to start the reaction). The increase in absorbance at 340 nm due to NADPH formation was followed for 9–12 min, and the linear slope of the reaction was used for calculation of HK activity. Protein concentrations of samples were determined using the Bio-Rad (Richmond, CA) protein assay (26), and enzyme activities are expressed as nanomoles per minute per milligram protein.

Other analyses. Plasma glucose was measured by a glucose oxidase method, immediately after sampling, using a Yellow Springs Instruments analyzer (YSI 2700; Yellow Springs, OH). To determine [3-^{3}H]glucose specific activity, 1 ml of plasma was deproteinized with $Ba(OH)_2/ZnSO_4$ (27). After centrifugation, the neutral supernatant was evaporated, and the residue was dissolved in 1 ml water. After adding 10 ml of scintillation fluid (Ecoscint, Manville, NJ), ^{3}H disintegrations per min were determined in an ICN 36014 liquid scintillation counter (Titertek Instruments, Huntsville, AL) using an external standard to correct for quenching. Quadruplicate aliquots of the labeled glucose infusate were added to nonradioactive plasma and processed in parallel with the plasma samples to allow calculation of the [3-^{3}H]glucose infusion rate.

Serum insulin was measured by a double antibody technique (28). The intra- and interassay coefficients of variation (CVs) were 6.8 and 7.9%, respectively. Urinary N excretion was calculated from the urine concentrations of creatinine, uric acid, and urea (20).

Calculation of glucose appearance rate (R_a) and R_d. In the basal state, R_a and R_d were calculated by dividing the [3-^{3}H]glucose infusion rate by the plasma glucose specific activity using the mean of the four basal plasma samples. During the clamp, R_a and R_d were calculated using the non–steady-state equations of Steele et al. (29). A distribution volume of 0.19 l/kg and a pool fraction of 0.5 were used in the calculation (30). In the NIDDM patients, basal R_d was corrected for urinary glucose excretion. Hepatic glucose output (HGO) was calculated by subtracting the exogenous glucose infusion rate from R_a.

Statistical analysis. Results are expressed as means ± SE unless otherwise indicated. The significance of differences within groups was tested by Student's paired t test and between groups by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Correlations were sought by Pearson's least-squares method. A P value < 0.05 was considered statistically significant.

RESULTS

Plasma glucose and insulin levels. Fasting plasma glucose concentrations were higher in the NIDDM patients (8.9 ± 0.9 mmol/l) than in the lean (5.1 ± 0.1 mmol/l) or obese (5.1 ± 0.1 mmol/l) control subjects ($P < 0.001$ for both). Fasting insulin levels were also higher in the NIDDM patients than in the lean control subjects (131 ± 30 vs. 21 ± 3 pmol/l, $P < 0.01$) but not significantly different from those of obese subjects (72 ± 22 pmol/l). In the NIDDM patients, a plasma glucose of 4.4 mmol/l was reached by 80 ± 43 (SD) min of the glucose clamp. Plasma glucose levels during the last 30 min of the clamp were similar in the three groups as was the CV of plasma glucose calculated for each subject (lean control subjects 4.6 ± 1.9 [SD], obese control subjects 3.6 ± 2.3, NIDDM patients 2.0 ± 1.4%). Steady-state insulin levels during the clamp were similar in the three groups (lean control subjects 915 ± 81, obese control subjects 931 ± 82, NIDDM patients 926 ± 44 pmol/l).

In the 10 lean subjects who participated in the meal study, fasting glucose levels were 4.9 ± 0.1 mmol/l, increasing to a peak of 7.2 ± 0.5 mmol/l 60 min after the start of the meal. Fasting insulin levels were 30 ± 9 pmol/l, increasing to a peak of 450 ± 51 pmol/l at 60 min after the start of the meal. The area under the 4-h plasma insulin curve after the meal was 1,064 ± 172 pmol/l · h.

R_a and R_d. In the basal state, R_a was higher in the NIDDM patients (18.2 ± 1.6) than in the lean (14.0 ± 1.0) or obese (10.9 ± 1.3 µmol · min^{-1} · kg^{-1}) control subjects. R_d, corrected for urinary glucose excretion, was also higher in the hyperglycemic NIDDM patients (18.0 ± 1.5) than in the obese subjects (10.9 ± 1.3 µmol · min^{-1} · kg^{-1}, $P < 0.05$) but was not statistically different from that of the lean control subjects. The somewhat

lower basal R_a and R_d in obese subjects were not statistically significantly different from those of the lean control subjects.

R_a for the 270- to 300-min interval of the glucose clamp was lower in the NIDDM patients (18.2 ± 1.9) than in the two control groups (lean 55.6 ± 3.5, obese 37.8 ± 6.1 μmol · min^{-1} · kg^{-1}, $P < 0.001$ and $P < 0.05$, respectively). HGO during this period was completely suppressed in the lean (0.9 ± 0.9) and obese (1.5 ± 1.0) control subjects but not in the NIDDM patients (4.6 ± 1.1 μmol · min^{-1} · kg^{-1}, $P < 0.01$ and $P < 0.05$ vs. lean and obese control subjects, respectively). In the lean normal subjects, R_d during the last 30 min of the clamp was increased fourfold over that in the basal state (Fig. 1). In the NIDDM patients, R_d showed little change from basal, and during the 150- to 180-min and 270- to 300-min intervals of the clamp, it was markedly reduced by comparison with the lean and obese control subjects (Fig. 1). This failure of R_d to rise during the clamp in the NIDDM patients is due, in part, to their reduction in plasma glucose levels during the clamp as well as their insulin resistance. During the last 30 min of the clamp, R_d in the NIDDM patients was only 18.1 ± 1.8 compared with 55.9 ± 3.4 in the lean ($P < 0.001$) and 37.7 ± 6.0 μmol · min^{-1} · kg^{-1} in the obese control groups ($P < 0.005$).

Substrate oxidation rates. After an overnight fast, carbohydrate and lipid oxidation rates were similar in the three groups (Fig. 1). In response to the glucose clamp, glucose oxidation increased less in the NIDDM patients than in the lean control subjects, while obese subjects had an increase in glucose oxidation that was intermediate between the other two groups (Fig. 1). Lipid oxidation was suppressed to <10% of the basal rate after 120 min in the lean and obese normal subjects. In NIDDM patients, suppression of lipid oxidation was impaired (Fig. 1), remaining at 62% of the basal rate during the last 3 h (0.20 ± 0.05), and during this period, it was significantly higher than in the lean (0.03 ± 0.03) or obese (0.01 ± 0.01 mg · min^{-1} · kg^{-1}) control subjects ($P < 0.05$ for both).

Nonoxidative R_d was higher in the basal state in the NIDDM patients than in the lean or obese control subjects (Fig. 1). This increase is explicable by their higher fasting glucose levels, which would enhance tissue glucose uptake and its metabolism to lactate and pyruvate by a mass action effect. During the clamp, nonoxidative R_d increased eightfold in lean subjects and ninefold in obese subjects, consistent with an increase in glucose deposition as muscle glycogen (1–3). In the NIDDM patients, nonoxidative R_d fell, and during the last 30 min of the clamp it was lower than in the basal state (Fig. 1, $P < 0.02$). This reduction is consistent with a marked defect of muscle glycogen deposition during the clamp in the NIDDM patients in combination with enhanced glucose oxidation that, given the unchanged R_d, may largely be secondary to the suppression of lipid oxidation (Fig. 1), which would allow a larger proportion of the pyruvate produced by glycolysis to be oxidized.

Skeletal muscle citrate synthase activity. Basal skeletal muscle citrate synthase activity (Table 1) was highest in the lean control subjects and lowest in the NIDDM patients. No change in citrate synthase activity was found in any of the groups in response to the glucose clamp. By repeated measures ANOVA, citrate synthase activity was lower in the NIDDM patients than in lean ($P < 0.001$) or obese normal subjects ($P < 0.05$), but the difference between obese and lean subjects did not reach statistical significance. Muscle citrate synthase activity showed a strong correlation with R_d at the end of the glucose clamp ($r = 0.734$, $P < 0.001$) (Fig. 2).

In the 10 normal subjects given a high-carbohydrate meal, muscle citrate synthase activity 4 h after the meal (67.7 ± 3.7) was unchanged from basal (66.4 ± 4.3 nmol · min^{-1} · mg^{-1} protein).

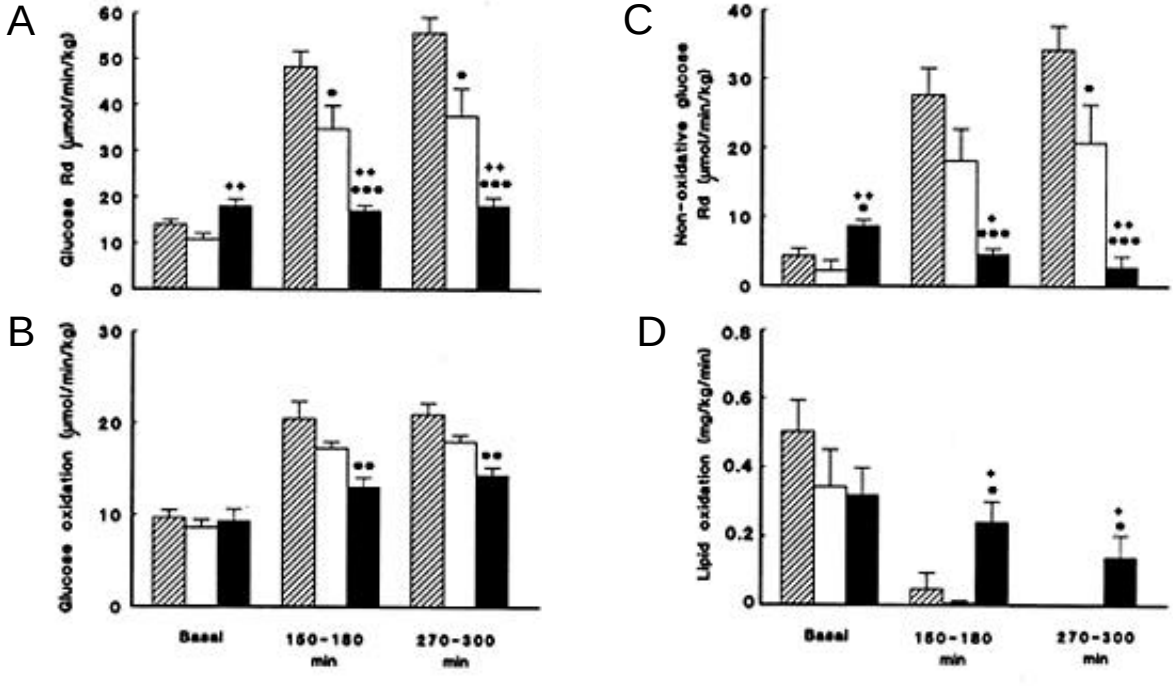

FIG. 1. R_d (A), glucose oxidation (B), nonoxidative R_d (C), and lipid oxidation (D) in seven lean normal subjects (▨), six obese normal subjects (□), and eight patients with NIDDM (■) in the basal state and between 150 and 180 min and 270 and 300 min of a 5-h 80 mU · m^{-2} · min^{-1} hyperinsulinemic euglycemic clamp. Values are expressed as means ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. lean control subjects; $^{+}P < 0.05$, $^{++}P < 0.005$ vs. obese control subjects.

TABLE 1
Skeletal muscle citrate synthase and HK activities after an overnight fast in lean and obese normal subjects and NIDDM patients

	Normal subjects		NIDDM patients
	Lean	Obese	
n	7	6	8
Citrate synthase	64.5 ± 5.0	55.8 ± 4.6	51.0 ± 1.4
Total HK	5.05 ± 0.53	4.75 ± 0.44	4.29 ± 0.33
HK-I	2.72 ± 0.35	2.59 ± 0.21	2.87 ± 0.25
HK-II	2.33 ± 0.24	2.16 ± 0.37	1.42 ± 0.16*
% HK-II	46.9 ± 3.0	44.3 ± 4.6	33.1 ± 2.8†

Data are means ± SE. Enzyme activities are expressed in nanomoles per minute per milligram of protein. *P < 0.05, †P < 0.025 compared with lean control subjects.

Skeletal muscle HK activity. In the basal state, lean and obese normal subjects had similar muscle activities of the two HK isoforms and of total HK (Table 1). HK-II accounted for 47 ± 3 and 44 ± 5% of total HK activity in the lean and obese subjects, respectively. Basal skeletal muscle HK-II activity was lower in the NIDDM patients than in the lean control subjects (Table 1, P < 0.05) and accounted for a lower proportion of muscle total HK activity (Table 1, P < 0.025). Total HK and HK-I activity did not, however, differ from those of control subjects. Total HK activity increased during the clamp in lean and obese subjects (Fig. 3). This was entirely due to an increase in HK-II, which, in the lean and obese subjects, was 34 and 36% higher than it was in the basal state after 3 h and 14 and 22% higher than it was in the basal state after 5 h of hyperinsulinemia (Fig. 3). By contrast, in the NIDDM patients, neither muscle HK-II nor total HK activity changed in response to the euglycemic clamp. Muscle HK-II activity during the clamp (average of activities in the 3- and 5-h biopsies) correlated with muscle citrate synthase activity in the 13 normal subjects (r = 0.58, P < 0.05) and in all three groups combined (r = 0.64, P < 0.002) (Fig. 4). A weak relationship was noted between muscle HK-II activity and R_d at the end of the clamp when the three groups were combined (r = 0.49, P < 0.05) (Fig. 4) but not in any of the groups considered separately.

In the 10 normal subjects who participated in the meal study, skeletal muscle HK-I activity was not different 4 h after the meal (3.26 ± 0.16) than in the overnight fasted state (3.15 ± 0.22 nmol · min^{-1} · mg protein^{-1}). However, HK-II activity increased 15% from 2.47 ± 0.19 to 2.86 ± 0.28 (P < 0.05) (Fig. 5), and total HK also tended to be higher (6.11 ± 0.37 vs. 5.62 ± 0.32 nmol · min^{-1} · mg protein^{-1}), although the difference did not reach statistical significance (t = 2.068, 0.05 < P < 0.1, NS).

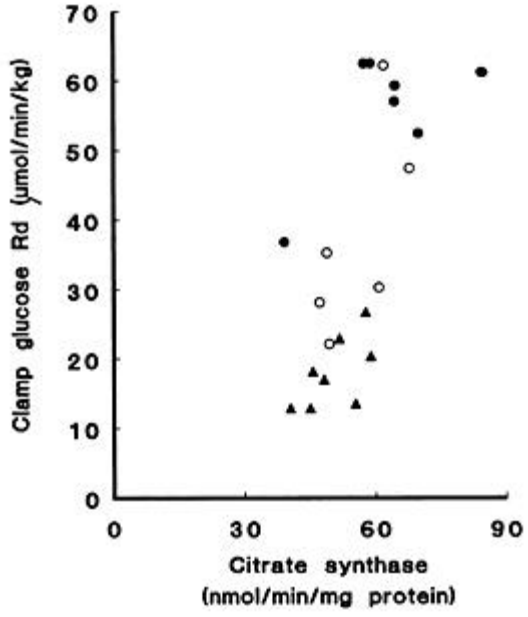

FIG. 2. Relationship between R_d during the last 30 min of the hyperinsulinemic glucose clamp and skeletal muscle citrate synthase activity in seven lean normal control subjects (●), six obese normal control subjects (○), and eight patients with NIDDM (▲). For the three groups combined, r = 0.734, P < 0.001.

DISCUSSION

Skeletal muscle is the main site of insulin resistance in obesity and NIDDM (1–4). ^{13}C-nuclear magnetic resonance studies showed that during a hyperinsulinemic glucose clamp, muscle G-6-P levels increase less in NIDDM patients than in normal subjects (31), consistent with a defect at the level of either glucose transport or phosphorylation. An impairment of muscle glucose transport in association with decreased recruitment of GLUT4 to the muscle plasma membrane is found in both obesity and NIDDM (11,32). Kelley et al. (11), using dynamic positron-emission tomography scanning, showed that in NIDDM there is an additional defect of glucose phosphorylation. This was also suggested by Bonadonna et al. (33). The finding that HK-II gene expression in skeletal muscle and L6 myotubes is regulated by insulin (9,10,14,16,34) and the finding that HK-II protein and activity are decreased in muscle of NIDDM patients (12), suggest that abnormal regulation of HK-II might contribute to the defect of muscle glucose phosphorylation found in NIDDM (11,33). Our aim was to test the hypothesis that basal and insulin-stimulated HK-II activity is decreased in insulin-resistant NIDDM patients and obese subjects and relate the activity of this enzyme to in vivo insulin action.

The NIDDM patients were very insulin resistant, with an R_d at the end of the 5-h glucose clamp that was only 32% of that in the lean control subjects. Both oxidative and nonoxidative R_d were markedly impaired (Fig. 1). The nondiabetic obese subjects were also insulin-resistant, with an R_d intermediate between that of the lean subjects and NIDDM patients (Fig. 1). In agreement with Vestergaard et al. (12), we found that after an overnight fast, muscle HK-II activity was 39% lower in NIDDM patients than in lean subjects (P < 0.05) and accounted for a lower proportion of total HK activity (33 ± 3 vs. 47 ± 3%, P < 0.025). Like Kelley et al. (11), we found that the insulin-resistant obese nondiabetic subjects had normal basal HK-II activity. Total and HK-I activity did not differ between the three groups, consistent with previous findings (11,12).

The contribution of HK-II to total HK activity in our lean subjects (47%) is much lower than the 79% reported by Mandarino et al. (14) but very similar to that found by others (12) and consistent with early studies that found more HK-I than HK-II protein in human skeletal muscle (35). It is notable that in the study of Mandarino et al., HK-II protein, as distinct from activity, accounted for only 55% of total HK protein in the basal state (14). This divergence between HK-II protein levels and activity found by Mandarino et al. is

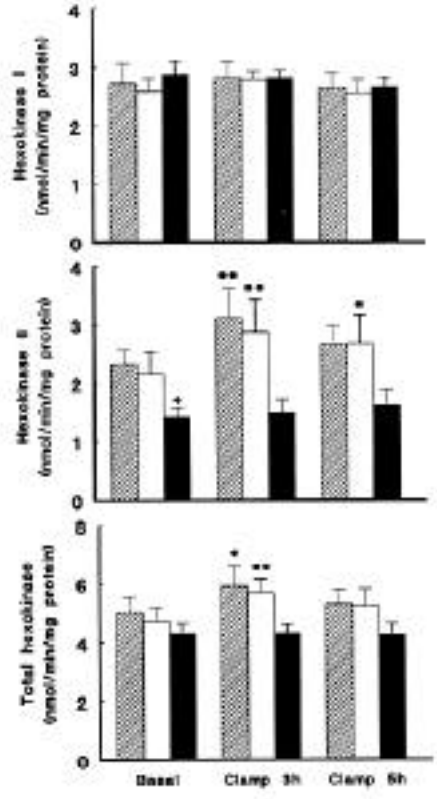

FIG. 3. HK-I, HK-II, and total HK activity in vastus lateralis muscle biopsies from seven lean control subjects (▨), six obese normal control subjects (□), and eight patients with NIDDM (■) after an overnight fast (basal) and at 3 and 5 h of an 80 mU · m^{-2} · min^{-1} hyperinsulinemic euglycemic clamp. Values are expressed as means ± SE. *P < 0.05, **P < 0.01 compared with activity in the basal state. ^{+}P < 0.05 compared with lean normal control subjects in the basal state.

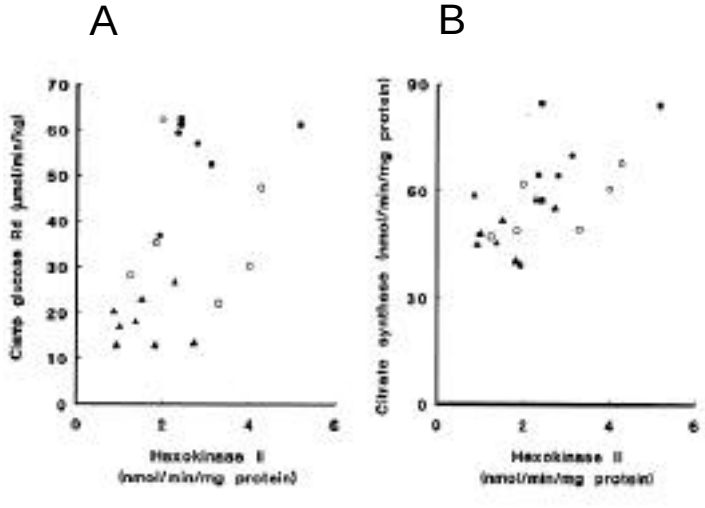

FIG. 4. A: Relationships between R_d during the last 30 min of the 5-h hyperinsulinemic glucose clamp and skeletal muscle HK-II activity in seven lean normal control subjects (●), six obese normal control subjects (○), and eight patients with NIDDM (▲). For the three groups combined, r = 0.49, P < 0.05. B: Relationships between citrate synthase activity and HK-II activity in skeletal muscle in the study subjects (same symbols). For the 13 normal subjects, r = 0.58, P < 0.05; for the three groups combined, r = 0.64, P < 0.002.

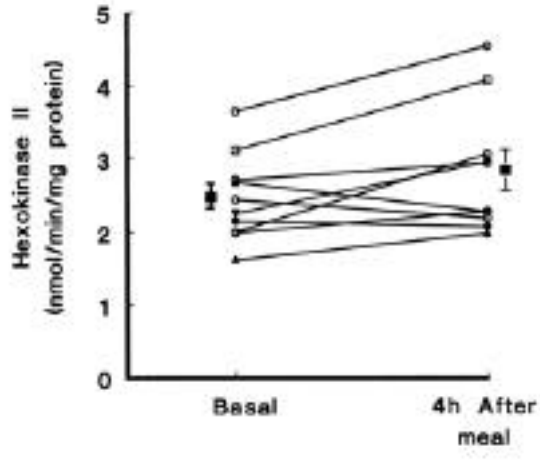

FIG. 5. Skeletal muscle HK-II activity in 10 lean normal subjects before and 4 h after the start of a high-carbohydrate meal. Values are expressed as means ± SE. HK-II activity was significantly higher after the meal than in the basal state (P < 0.05).

unexplained. Their data suggest that HK-II enzyme-specific activity is higher than that of HK-I.

HK-II (but not HK-I) activity increased during the clamp in lean and obese subjects by 34 and 36% after 3 h and by 14 and 22% after 5 h of hyperinsulinemia; no increase was found in the NIDDM patients (Fig. 3). The lower HK-II activity in NIDDM patients, and the increase during the clamp in normal subjects, are probably due to a change in HK-II enzyme content, since in other studies, changes in HK-II activity are accompanied by qualitatively similar changes in HK-II mRNA and protein levels (6,9,12,14). The insulin-induced increase in muscle HK-II activity in the lean control subjects is comparable to that found by Mandarino et al., who used a lower insulin infusion rate (40 mU · m^{-2} · min^{-1}) (14). They found a threefold increase in HK-II mRNA and a 59% increase in cytosolic HK-II activity, which corresponds to an increase in total homogenate HK-II activity of about 29%, as 67% of HK-II in their muscle samples was in a crude mitochondrial fraction, and activity in this fraction did not change in response to insulin. In another study, they found that insulin increased cytosolic and total homogenate HK-II activity by about 39 and 11%, respectively (36), while others found no effect of insulin on muscle HK-II activity (11).

Our finding in lean normal subjects that skeletal muscle HK-II activity increased by 15% (P < 0.05) within 4 h of a large carbohydrate-rich meal (Fig. 5) suggests that the response to insulin seen during a glucose clamp is physiologically relevant. Although this increase may seem small, if further increments occurred with additional meals during the day, one might expect a diurnal variation in muscle HK-II activity. Muscle HK-II activity falls in rodents with prolonged fasting (37), suggesting that the hormonal/nutrient changes associated with eating may be important determinants of basal HK-II activity. In our NIDDM patients, basal muscle HK-II activity was reduced despite fasting insulin levels that were much higher than those in the lean control subjects, suggesting resistance to insulin, in keeping with the lack of stimulation of HK-II expression by insulin during the glucose clamp (Fig. 3).

The finding of normal basal and insulin-stimulated HK-II activity in the obese subjects, who are at increased risk for

NIDDM, suggests that the defect in muscle HK-II expression in NIDDM may be secondary to the diabetic state. This fits with the finding that muscle HK-II activity is decreased in the streptozotocin-induced diabetic rat and is restored by insulin therapy (7,16). The relative roles of insulin resistance, relative insulin deficiency, and poor diabetic control in causing the reduced muscle HK-II activity are unclear; all may be important. Vestergaard et al. (12) found that skeletal muscle HK-II activity in NIDDM patients was inversely related to both plasma glucose and nonesterified fatty acid levels. The finding in the obese insulin-resistant KKA^{Y} diabetic mouse that decreased muscle HK-II mRNA abundance was partially restored by pioglitazone treatment, which improved glucose control without increasing insulin levels (6), also suggests that poor diabetic control may be important.

Glucose transport is thought to be rate-limiting for glucose utilization in skeletal muscle under most circumstances (38–40), but at high glucose flux rates, as with insulin stimulation, or in transgenic mice that overexpress GLUT1 in skeletal muscle, glucose phosphorylation (hence HK activity) may become rate-limiting (41,42). In L6 myotubes, increased HK-II mRNA and protein levels are associated with increased glucose utilization rates (34). Similarly, transgenic mice that overexpress HK-II in skeletal muscle have increased 2-deoxyglucose uptake and phosphorylation at both submaximally and maximally stimulating concentrations of insulin (43). Taken together, these observations suggest that the level of HK-II expression in muscle is an important determinant of muscle insulin sensitivity. The relationship between muscle HK-I and HK-II protein levels/enzyme activity as measured in vitro and the capacity for muscle glucose uptake and phosphorylation is not, however, straightforward. A proportion of HK-I and HK-II is bound to porin of the outer mitochondrial membrane (13). HK associated with mitochondria is much less sensitive to inhibition by G-6-P than cytosolic HK (13). Indeed, cytosolic HK is thought to be mostly inhibited, since the K_i of soluble HK for G-6-P is in the low micromolar range (44), while cytosolic G-6-P concentration is between 0.1 and 0.3 mmol/l (31). Thus, in vivo activity is probably determined primarily by enzyme bound to mitochondria. Mandarino et al. found 60–70% of HK-II but <20% of HK-I in a crude mitochondrial fraction of human muscle (14), even though HK-I in other tissues does associate with mitochondria (13,45). If this difference in the distribution of the two isozymes in human muscle is confirmed, it implies that HK-II, rather than HK-I or total HK, is the main determinant of glucose phosphorylation capacity, and it suggests that a 39% reduction in HK-II activity in NIDDM patients could contribute to insulin resistance even though HK-I activity is maintained.

Normal HK-II activity in the obese normal subjects, but reduced HK-II in NIDDM patients, fits with the finding of a quantitatively similar defect of glucose transport and GLUT4 translocation in muscle of NIDDM patients and obese normal subjects in response to insulin during a glucose clamp and an additional defect of glucose phosphorylation only in NIDDM patients (11). Thus, the lesser impairment of insulin-stimulated muscle glucose uptake in obesity compared with NIDDM, and the normal maximal glucose disposal rates in obese subjects with less severe insulin resistance (1,46), might be explained by their normal HK-II activity and, hence, normal capacity for glucose phosphorylation. The weak relationship between muscle HK-II activity and insulin sensitivity (Fig. 4) is perhaps to be expected given that the location of the rate-limiting step for glucose utilization (transport versus phosphorylation) may depend on where an individual is on the glucose uptake–insulin dose response curve. A better correlation might be expected between HK-II activity and maximal R_d, since HK activity appears to provide a quantitative index of the potential maximum rate of glucose utilization (47).

Citrate synthase is a mitochondrial enzyme whose activity in muscle correlates with mitochondrial content and oxidative capacity (48). Previous studies suggested decreased skeletal muscle oxidative capacity in NIDDM and in obesity that correlated with insulin sensitivity (49–51). Muscle citrate synthase activity was significantly lower in the NIDDM patients than in the lean or obese control subjects, suggesting a reduced muscle oxidative capacity. Like others, we found a good correlation ($r = 0.73$, $P < 0.001$) between citrate synthase activity and R_d during the clamp (Fig. 2) (49). Muscle citrate synthase did not change during the clamps in any group and was unaffected by ingestion of a high-carbohydrate meal in the lean control subjects, consistent with the lack of any known acute effect of insulin on this enzyme. Citrate synthase and HK activities vary with muscle fiber type; highest activities are found in skeletal muscles with a predominance of type I oxidative fibers and in the heart (52,53). This may explain the positive correlation found between muscle HK-II and citrate synthase activity ($r = 0.64$, $P < 0.002$) (Fig. 4). A reduced number of type I fibers has been reported in obesity (54,55) and in obese and nonobese NIDDM patients (56,57), although others found normal muscle fiber composition in NIDDM (32). Simoneau et al. (49) suggested that the lower citrate synthase activity/muscle oxidative capacity in insulin-resistant subjects is independent of muscle fiber–type composition. Further studies are thus needed to determine whether the abnormalities of citrate synthase and HK-II in our insulin-resistant subjects are related to alterations in muscle fiber composition.

In summary, the NIDDM patients had lower muscle HK-II activity basally and did not increase the activity of this enzyme in response to a 5-h insulin stimulus. They also had reduced muscle citrate synthase activity, suggesting a lowered oxidative capacity of skeletal muscle. These defects may contribute to their insulin resistance. In the nondiabetic obese subjects, muscle HK-II expression and its regulation by insulin was normal, which may be important in determining their better insulin action on glucose disposal by comparison with the NIDDM patients.

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