

Insulin-Stimulated Akt Kinase Activity Is Reduced in Skeletal Muscle From NIDDM Subjects

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The serine/threonine kinase Akt (PKB/Rac) has been implicated as playing a role in the insulin-signaling pathway to glucose transport. Little is known regarding the regulation of Akt kinase activity in insulin-sensitive tissues, such as skeletal muscle, or whether this regulation is altered in insulin-resistant states such as NIDDM. We examined the effect of insulin on Akt kinase activity in skeletal muscle from six NIDDM patients and six healthy subjects. Whole-body insulin sensitivity, assessed by the euglycemic-hyperinsulinemic clamp, was significantly lower in NIDDM subjects ($P < 0.001$), and this was accompanied by impaired *in vitro* insulin-stimulated glucose transport in skeletal muscle. In both groups, insulin induced a significant increase in Akt kinase activity, but the response to maximal insulin (60 nmol/l) was markedly reduced in skeletal muscle from NIDDM subjects (66% of control levels, $P < 0.01$). Impaired Akt kinase activity was not accompanied by decreased protein expression of Akt. Instead, a trend toward increased Akt expression was noted in skeletal muscle from NIDDM subjects ($P < 0.1$). These parallel defects in insulin-stimulated Akt kinase activity and glucose transport in diabetic skeletal muscle suggest that reduced Akt kinase activity may play a role in the development of insulin resistance in NIDDM. *Diabetes* 47:1281–1286, 1998

Although the primary defect responsible for the development of NIDDM is unknown, a combination of genetic and environmental factors contribute to the manifestation of this progressive metabolic disorder (1,2). Reduced whole-body insulin responsiveness is a characteristic feature of NIDDM patients. Thus, people with NIDDM demonstrate decreased ability to dispose glucose during a euglycemic-hyperinsulinemic clamp compared with healthy individuals (3). Skeletal muscle is a primary site of insulin resistance in NIDDM (4), and we and others have previously shown that insulin-

stimulated glucose transport is severely blunted in skeletal muscle from NIDDM subjects compared with control subjects (5–7).

The insulin-signaling pathway has been the subject of intense interest, which has resulted in an increased understanding of the molecular mechanisms involved in signaling pathways to glucose transport. Early and intermediate steps in the insulin-signaling cascade are candidates for defects leading to insulin resistance in skeletal muscle. Indeed, impaired insulin receptor kinase activity has been noted in skeletal muscle from subjects with NIDDM and obesity (8). We have previously shown that insulin receptor substrate-1 tyrosine phosphorylation and phosphatidylinositol (PI) 3-kinase activity are severely impaired in skeletal muscle from NIDDM subjects after *in vivo* insulin infusion (9). Similar defects in insulin stimulation of insulin receptor substrate-1 phosphorylation and PI 3-kinase activity were noted in skeletal muscle from severely obese insulin-resistant subjects (10). However, the extent to which these signaling defects are causative in the development of insulin resistance or secondary to the altered metabolic state associated with NIDDM is currently not known.

Akt (also termed PKB or Rac) is a 60-kDa serine/threonine kinase, which is stimulated by a wide range of receptor tyrosine kinases (11–15). Akt is thought to be a downstream target of PI 3-kinase (12,13,16), which is activated presumably through activation of the recently identified phosphatidylinositol 3,4,5-trisphosphate-dependent protein kinase (PDK1) (17). Several lines of evidence suggest that PI 3-kinase is both necessary and sufficient for growth factor-dependent activation of Akt kinase activity (11–13,15). Furthermore, Akt is thought to play an important role in regulation of cell survival (18–21), and it has also been implicated in the signaling pathway to glucose transporter translocation (22,23). Importantly, expression of constitutively active Akt is sufficient to promote GLUT4 translocation and increase glucose transport in 3T3-L1 adipocytes (22,23). Thus, Akt is likely to play an integral role in the insulin-signaling cascade to glucose transport.

We have recently shown that insulin stimulation of Akt is significantly decreased in the nonobese spontaneously diabetic Goto-Kakizaki (GK) rat compared with nondiabetic animals (24). When hyperglycemia was normalized by 4 weeks phlorizin treatment, glucose tolerance and insulin-stimulated glucose transport was improved. Furthermore, insulin stimulation of Akt kinase was restored to control levels (24). Thus, in a nonobese animal model of NIDDM, insulin-stimulated Akt kinase activity and glucose transport were reduced in parallel in skeletal muscle. Furthermore, both defects were reversed following improved glycemic control.

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ANOVA, analysis of variance; BSA, bovine serum albumin; DTT, dithiothreitol; GSK-3, glycogen synthase kinase 3; KHB, Krebs-Henseleit Buffer; PDK1, phosphatidylinositol 3,4,5-trisphosphate-dependent protein kinase; PI, phosphatidylinositol; PKB, protein kinase B; Rac, related to A and C protein kinase.

TABLE 1
Subject characteristics

	NIDDM patients	Control subjects
<i>n</i>	6	6
Age (years)	55 ± 3	55 ± 3
BMI (kg/m ²)	27.4 ± 1.8	26.3 ± 0.8
Fasting glucose (mmol/l)	8.9 ± 1.0*	5.4 ± 0.1
Fasting insulin (pmol/l)	97 ± 20†	36 ± 8
HbA _{1c} (%)	6.9 ± 0.4§	4.7 ± 0.1
Duration of diabetes (years)	7.4 ± 2.3	—

Data are means ± SE. **P* < 0.05, †*P* < 0.02, §*P* < 0.001, significantly different versus control subjects.

Whether defects in Akt kinase activity contribute to reduced glucose transport in skeletal muscle in insulin-resistant humans remains to be elucidated.

Here we report that insulin induces an increase in Akt kinase activity in human skeletal muscle. Furthermore, insulin-stimulated Akt kinase activity in skeletal muscle from NIDDM subjects was significantly reduced compared with control subjects, and this reduction was not linked to a reduced protein expression of Akt.

RESEARCH DESIGN AND METHODS

Subject characteristics. The study protocol was reviewed and approved by the institutional ethical committee of the Karolinska Institute, and informed consent was received from all subjects before participation. The clinical characteristics of the subjects are presented in Table 1. The diabetic group consisted of six male NIDDM patients with a mean duration of disease of 7.5 years, ranging from newly diagnosed to 15 years. Glycemic control, as evaluated by HbA_{1c}, was moderate (6.9 ± 0.4%). The normal range for HbA_{1c} in our laboratory is <5.2%. One subject was treated with insulin alone, one with a combination of sulfonylureas and insulin, two with sulfonylureas, and two with diet alone. The control group consisted of six healthy male subjects. None of the study participants were smokers or were taking any other medication. The subjects were instructed to abstain from any form of strenuous physical activity for a period of 48 h before the experiment. On the day of the test, the subjects reported to the laboratory after an overnight fast, and in the case of the NIDDM patients, before administration of any antidiabetic medication.

Euglycemic-hyperinsulinemic clamp procedure. Peripheral insulin sensitivity was evaluated by the euglycemic-hyperinsulinemic clamp procedure (25). A polyethylene catheter was placed in the brachial artery for blood determinations. A second catheter was placed in an antecubital vein for glucose and insulin infusions. A bolus infusion of insulin was given for 5 min (18.4 nmol · kg⁻¹ · min⁻¹). The infusion rate was then reduced for 5 min (9.2 nmol · kg⁻¹ · min⁻¹), followed by a continuous infusion of insulin (6.9 nmol · kg⁻¹ · min⁻¹) for 2 h to obtain physiological hyperinsulinemia. The fasting plasma glucose concentration was maintained throughout the insulin infusion by means of a variable glucose infusion and blood glucose determinations every 5 min. Whole-body peripheral glucose utilization was calculated during the last 100-min period of the steady-state insulin infusion.

Open muscle biopsy procedure and in vitro incubation of human skeletal muscle. Open muscle biopsies were performed as described previously (26). Muscle biopsies were obtained from one healthy control subject and one NIDDM patient on the same day. Muscle specimens from these individuals were processed in parallel. Briefly, biopsies were obtained under local anesthesia from the vastus lateralis portion of the quadriceps femoris muscle and placed in oxygenated Krebs-Henseleit buffer (KHB), which contained 5 mmol/l HEPES and 0.1% bovine serum albumin (BSA) (RIA Grade; Sigma, St. Louis, MO). Smaller muscle strips were dissected, mounted on Plexiglass clamps, and incubated in vitro in pre-gassed (95% O₂/5% CO₂) KHB as described previously (27). The gas phase in the vials was maintained at 95% O₂/5% CO₂ throughout all incubations. The muscles were incubated at 35°C for 30 min in KHB containing 5 mmol/l glucose and 15 mmol/l mannitol, and subsequently with or without insulin (0.6, 2.4, or 60 nmol/l, as indicated) for 20 min.

Glucose transport activity. Muscles were next incubated at 35°C with or without insulin (0.6, 2.4, or 60 nmol/l as indicated) for an additional 20 min in KHB containing 5 mmol/l [³H]3-*O*-methylglucose (2.5 μCi/mmol) and 15 mmol/l [¹⁴C]mannitol (26.3 μCi/mmol). The total insulin exposure time was thus 40 min.

Thereafter, the incubated muscle specimens were homogenized in ice-cold homogenizing buffer (50 mmol/l HEPES, pH 7.6, 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l Na₂VO₄, 10 mmol/l NaF, 30 mmol/l Na₂O₇, 10% [vol/vol] glycerol, 1 mmol/l benzamide, 1 mmol/l dithiothreitol [DTT], 10 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 μmol/l microcystin) and centrifuged at 150,000g for 35 min (4°C). Protein was determined using a commercial kit (Bio-Rad, Richmond, CA). Glucose transport was assessed as described by Wallberg-Henriksson et al. (28) and expressed as nmol 3-*O*-methylglucose · mg⁻¹ protein · h⁻¹. Duplicate samples were incubated under basal or insulin-stimulated conditions to assess glucose transport and Akt kinase activity (see below). When sufficient material was available, muscle was also incubated at 0.6 nmol/l insulin for glucose transport determinations.

Akt kinase activity and protein expression. From the remaining homogenate described above, aliquots of the supernatant (600 μg) were immunoprecipitated with anti-Akt-α antibody, and Akt kinase activity was measured against a peptide substrate (GRPRTSSFAEG) based on a motif from glycogen synthase kinase 3 (GSK-3) (29). The Akt-α antibody was raised in rabbit, against a fusion protein of the PH domain of human Akt-α and GST, which recognizes primarily the Akt-α (Akt1) isoform (30). Briefly, Akt immunoprecipitates were collected on protein-A sepharose beads and washed four times in buffer A (25 mmol/l HEPES, 10% glycerol, 1% Triton X-100, 1 mol/l NaCl, 1 mmol/l DTT, 0.1% BSA) and twice in kinase buffer (50 mmol/l Tris-HCl, pH 7.5, 10 mmol/l MgCl₂, 1 mmol/l DTT). They were re-suspended in 30 μl of kinase buffer supplemented with 5 μmol/l ATP, 100 μmol/l GSK-3 peptide, and 2 μCi [³²P]ATP. The reaction was terminated after a 30-min incubation at 30°C, and ³²P incorporation into the peptide substrate was determined by resolving the reaction products on a 40% acrylamide gel. The gel was visualized on a phosphorimager (Bio-Rad), and the band corresponding to the peptide substrate was quantitated. Results are presented as arbitrary phosphorimager units (PSL).

Aliquots (30 μg) of the supernatant were solubilized in Laemmli sample buffer, separated by SDS-PAGE (10% resolving gel), and transferred to nitrocellulose membranes. Immunodetection of Akt kinase protein was performed using the polyclonal Akt-α antibody described above. The nitrocellulose sheets were washed and incubated with appropriate secondary antibodies. Akt kinase protein was visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantified by densitometry.

Statistical analysis. Data are presented as mean ± SE. Samples from one NIDDM and one healthy control subject were processed for each analysis in parallel. Statistical differences were determined by analysis of variance (ANOVA) or by paired or unpaired *t* test, as appropriate.

RESULTS

Whole-body glucose utilization. The characteristics of the six NIDDM patients and the six healthy control subjects who participated in this study are given in Table 1. Age and

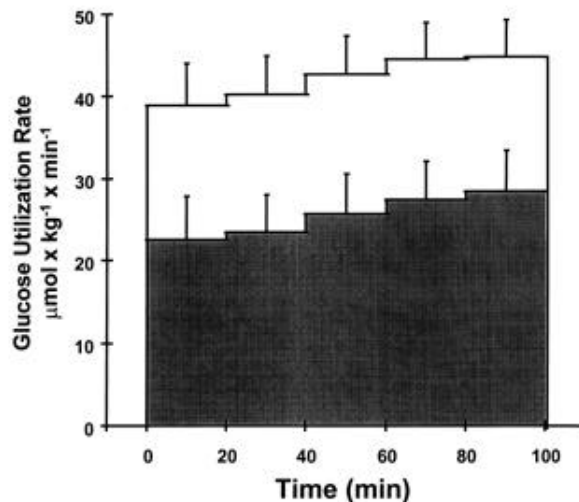


FIG. 1. Glucose infusion rate in NIDDM (■) and control (□) groups during an euglycemic-hyperinsulinemic clamp. Values are expressed as means ± SE. The glucose utilization rates were compared using a two-way ANOVA and found to be significantly different (*P* < 0.001).

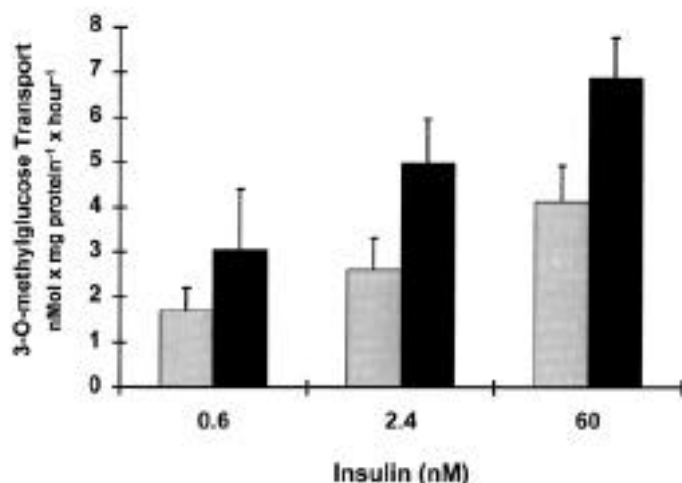


FIG. 2. Insulin-stimulated increment of 3-*O*-methylglucose transport in isolated skeletal muscle from NIDDM patients (■) and control subjects (▨). Muscle strips were incubated at 35°C for 40 min in the absence or presence of 0.6, 2.4, or 60 nmol/l insulin, as indicated. 3-*O*-Methylglucose transport was assessed as described in METHODS. Results are expressed as means \pm SE. The insulin response was compared using a two-way ANOVA and found to be significantly different ($P = 0.022$).

BMI were similar between the two groups. Figure 1 shows the glucose infusion rate in the NIDDM and the healthy control subjects during the euglycemic-hyperinsulinemic clamp. The mean peripheral glucose utilization rate achieved during steady-state hyperinsulinemia was reduced by 37% in the NIDDM patient group compared with the control group (25.8 ± 4.8 vs. $41.0 \pm 4.2 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $P < 0.001$ by ANOVA). The mean steady-state insulin levels during the last 100 min of the clamp were similar between the two groups (700 ± 43 pmol/l in NIDDM subjects vs. 609 ± 37 pmol/l in controls; NS). Mean blood glucose levels measured during steady state did not differ between the groups (5.3 ± 0.1 vs. 5.4 ± 0.1 mmol/l for NIDDM patients vs. healthy individuals, respectively; NS).

Skeletal muscle glucose transport. Insulin stimulation of 3-*O*-methylglucose transport was determined in the incubated muscle (Fig. 2). In control subjects, insulin stimulation resulted in a significant and dose-dependent increase in glucose transport, achieving a maximal 5.2 ± 0.7 -fold increase in response to 60 nmol/l insulin ($P < 0.01$). This response was markedly and significantly blunted in muscle from NIDDM patients ($P = 0.022$ by ANOVA compared with control response).

Akt kinase activity and protein expression. We next assessed whether insulin was able to stimulate the Akt kinase activity in human skeletal muscle. Insulin induced a maximal 10-fold increase in Akt kinase activity in muscle from control subjects (Fig. 3). At 2.4 nmol/l insulin, no difference in the ability of insulin to signal to Akt was observed between healthy control subjects and NIDDM patients. However, at 60 nmol/l insulin, Akt kinase activity was reduced by 34% in muscle from NIDDM patients compared with control subjects ($P < 0.01$). The amount of Akt protein present in the immunoprecipitations used to determine Akt kinase activity was similar between NIDDM and control subjects (data not shown).

Protein content of Akt was assessed in total muscle lysates to determine whether the reduced insulin-stimulated Akt kinase activity observed in NIDDM subjects was due to changes in Akt protein expression. Initial analysis of muscle lysate from five NIDDM subjects and five control subjects from the present study (due to insufficient material, Akt expression could not be assessed on one subject from each group) revealed a trend toward increased Akt expression in the NIDDM subjects. To pursue this further, we analyzed muscle lysate from an additional six NIDDM subjects and six control subjects. These subjects were similar in age (55 ± 3 for control subjects and 56 ± 3 for NIDDM subjects) and BMI (27.5 ± 1.2 and $27.1 \pm 2.0 \text{ kg/m}^2$, respectively) to the subjects in the present study. The characteristics of these subjects have been presented previously (9). The levels of Akt protein expression appeared to be quite variable between subjects. Akt expression level was not significantly different between control and NIDDM muscle, although we observed a trend toward increased expression in the NIDDM subjects ($P < 0.1$; Fig. 4).

DISCUSSION

Here we show that insulin significantly increases Akt kinase activity in human skeletal muscle. To our knowledge, this is the first evidence for insulin stimulation of Akt kinase activity in insulin-sensitive tissue from humans. This extends our previous observations of insulin stimulation of Akt phosphorylation in human skeletal muscle (31). Furthermore, for the first time, we show impaired insulin signaling at the level of Akt in skeletal muscle from insulin-resistant patients with NIDDM. The defect in insulin action on Akt kinase activity does not appear to be related to reduced protein expression of Akt; rather, Akt protein expression tended to be increased in skeletal muscle from NIDDM.

We have recently shown that insulin-stimulated Akt kinase activity in skeletal muscle from GK diabetic rats was markedly reduced as compared with control Wistar rats (24). After 4 weeks of phlorizin treatment, fasting hypergly-

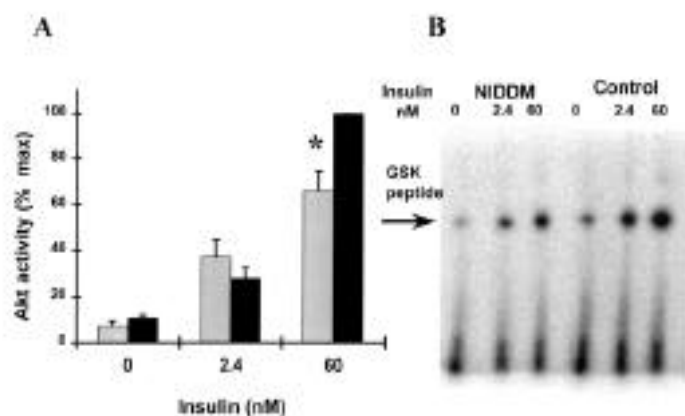


FIG. 3. **A:** Insulin-stimulated Akt kinase activity in skeletal muscle from NIDDM patients (■) and control subjects (▨). Isolated muscle was incubated as described in Fig. 2. Akt kinase activity in muscle lysates was measured against a peptide substrate based on a motif from GSK-3, as described in METHODS. Results are expressed as means \pm SE. Values are reported as percent of the insulin-stimulated value observed in the control subject of each analysis pair. *Significant difference from control muscle ($P < 0.01$). **B:** A representative autoradiogram of a phosphopeptide gel. The arrow indicates the position of the GSK peptide.

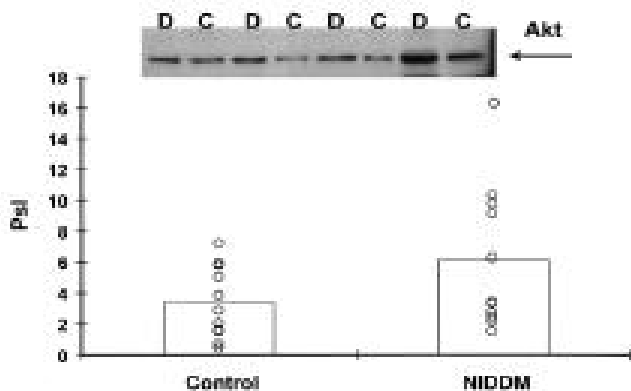


FIG. 4. Akt- α protein expression in skeletal muscle from 11 NIDDM patients and 11 control subjects. Muscle samples were prepared as described in METHODS. An aliquot of the lysate (30 μ g) was subjected to SDS-PAGE and immunoblotted with anti-Akt- α antibody. A representative autoradiograph is presented as an inset. Individual results are presented as open circles. PSl, arbitrary phosphorimager units; C, control; D, NIDDM.

cemia had been normalized in GK rats, and this was accompanied by a significant improvement in glucose tolerance and a complete normalization of insulin-stimulated Akt activity (24). In parallel to the present report in humans, at submaximal insulin concentrations, there was no difference in insulin-stimulated Akt kinase activity in skeletal muscle between diabetic and control rats. Taken together with the present observations in humans, chronically elevated glucose levels may lead to an impaired capacity for maximal insulin-stimulated Akt kinase activity in skeletal muscle. As 60 nmol/l insulin represents a super-physiological insulin concentration, the physiological relevance of this defect remains unclear. The approximately twofold increase in Akt protein levels observed in diabetic muscle may be a compensatory upregulation to maintain insulin-stimulated Akt kinase activity levels. Thus, insulin-stimulated Akt kinase activity per protein would appear to be impaired in diabetic muscle, even at lower insulin concentrations. At higher insulin concentrations, the observed upregulation in protein expression may not be sufficient to compensate for the impairment in insulin signaling.

A growing body of evidence suggests that chronic hyperglycemia directly contributes to the development of peripheral insulin resistance (32–38). For example, in partially pancreatectomized diabetic rats, correction of hyperglycemia by phlorizin treatment restored insulin-stimulated glucose transport in isolated adipocytes and improved whole-body insulin sensitivity (33). Whether a similar defect is present in human skeletal muscle exposed to chronic hyperglycemia is presently not known. We have previously shown that restoration of glycemia normalizes the reduced capacity for insulin-stimulated glucose transport in skeletal muscle from NIDDM patients (7). Thus, hyperglycemia may directly contribute to the development of insulin resistance in NIDDM patients through alterations at the level of insulin signaling in peripheral tissues (7,26,37). Whether normalization of glycemia is able to restore insulin signaling to Akt in NIDDM patients remains to be determined.

The activation of Akt has been closely linked to PI 3-kinase signaling (12,13,16), and as such, intense interest has

focused on the role of this kinase in the mediation of downstream growth factor-induced metabolic and mitogenic events (11–17). Activation of PI 3-kinase, either by growth factors (11–13,16,19) or by membrane localization (14,15), is accompanied by activation of Akt. Importantly, growth factor-induced activation of Akt is inhibited by pharmacological agents known to inhibit PI 3-kinase activity (11–13). These studies have all pointed to a role for Akt as a downstream mediator of PI 3-kinase-dependent signaling. This notion has been further strengthened by studies showing that overexpression of a constitutively active PI 3-kinase leads to increased activation of Akt (14,15), whereas overexpression of dominant-negative mutants of the p85 regulatory subunit of PI 3-kinase prevents stimulation of Akt kinase activity by platelet-derived growth factor (12). Recent studies have shown Akt to be activated by insulin in primary insulin-sensitive tissues, including isolated rat adipocytes (39,40) and rat skeletal muscle (24,40,41). Interestingly, in rat skeletal muscle, insulin increases Akt kinase activity in a time course that parallels the activation of PI 3-kinase (41).

Akt has also been linked to glucose transport in insulin-sensitive tissues. Overexpression of Akt in 3T3-L1 adipocytes directly promotes glucose transport and translocation of GLUT1 and GLUT4 to the plasma membrane (22,23), indicating that Akt may be sufficient to promote glucose transport. Activation of Akt kinase activity is also linked to PI 3-kinase-independent pathways. Okadaic acid mediates both the translocation of GLUT4 to the plasma membrane and activation of Akt, both via PI 3-kinase-independent mechanisms (42). Furthermore, there is also evidence that the activated form of p21ras will activate Akt (16,43). Osmotic shock stimulates glucose transporter translocation independent of PI 3-kinase activation. Osmotic shock has been shown to result in increased Akt kinase activity in Cos-7 cells (44), although this was not observed in 3T3L1 adipocytes (45). Thus, Akt may be a point of convergence for PI 3-kinase-dependent and -independent generated signals to glucose transporter translocation. However, whether Akt is linked to glucose transporter translocation remains to be firmly established. A definitive answer regarding the role of Akt in signaling to glucose transporter translocation would require the use of a specific pharmacological inhibitor of Akt or a dominant negative Akt construct.

Little is known regarding the physiologically relevant downstream targets of Akt. In the present study, we used a peptide substrate based on a motif from GSK-3 to assess Akt kinase activity. Akt has been implicated in playing a role in insulin signaling to the stimulation of glycogen synthesis by mediating the insulin-induced phosphorylation and inhibition of GSK-3 (30,40). Akt is likely to have other physiological substrates and has also been shown to activate p70^{S6} kinase (12) and phosphorylate and inactivate the apoptotic signaling protein BAD (20).

In conclusion, we provide the first evidence that insulin-stimulated Akt kinase activity is significantly reduced in skeletal muscle from insulin-resistant NIDDM subjects compared with age- and BMI-matched healthy control subjects. Taken together with our previous observations that long-term normalization of glycemia in a diabetic animal model restores insulin-stimulated Akt kinase activity, this finding suggests that hyperglycemia may have deleterious effects on intermediate and final components of the insulin-signaling

pathway in skeletal muscle. Whether the decrease in insulin-stimulated glucose transport in skeletal muscle from NIDDM patients is directly coupled to a defect in insulin action on Akt remains to be investigated.

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