Insulin-Stimulated Phosphorylation of the Akt Substrate AS160 Is Impaired in Skeletal Muscle of Type 2 Diabetic Subjects

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AS160 is a newly described substrate for the protein kinase Akt that links insulin signaling and GLUT4 trafficking. In this study, we determined the expression of and in vivo insulin action on AS160 in human skeletal muscle. In addition, we compared the effect of physiological hyperinsulinemia on AS160 phosphorylation in 10 lean−to−moderately obese type 2 diabetic and 9 healthy subjects. Insulin infusion increased the phosphorylation of several proteins reacting with a phospho-Akt substrate antibody. We focused on AS160, as this Akt substrate has been linked to glucose transport. A 160-kDa phosphorylated protein was identified as AS160 by immunoblot analysis with an AS160-specific antibody. Physiological hyperinsulinemia increased AS160 phosphorylation 2.9-fold in skeletal muscle of control subjects ($P < 0.001$). Insulin-stimulated AS160 phosphorylation was reduced 39% ($P < 0.05$) in type 2 diabetic patients. AS160 protein expression was similar in type 2 diabetic and control subjects. Impaired AS160 phosphorylation was related to aberrant Akt signaling; insulin action on Akt Ser^{473} phosphorylation was not significantly reduced in type 2 diabetic compared with control subjects, whereas Thr^{308} phosphorylation was impaired 51% ($P < 0.05$). In conclusion, physiological hyperinsulinemia increases AS160 phosphorylation in human skeletal muscle. Moreover, defects in insulin action on AS160 may impair GLUT4 trafficking in type 2 diabetes. *Diabetes* **54:1692–1697, 2005**

Peripheral insulin resistance is a major clinical feature of type 2 diabetes. Insulin resistance in skeletal muscle from type 2 diabetic patients is associated with impaired signal transduction at the level of insulin receptor substrate 1 (IRS-1) and phosphatidylinositol (PI) 3-kinase (1–4) and glucose transport (1,2,5). Akt is a downstream effector of PI 3-kinase that is directly linked to the regulation of glucose transport (6). Although defects in IRS-1/PI 3-kinase and glucose transport have been observed in several cohorts of type 2 diabetic subjects, impairments at the level of the Ser/Thr kinase Akt have been less apparent (rev. in 7). The role of Akt in skeletal muscle insulin resistance has been a challenge to resolve due to the presence of multiple isoforms that offer compensatory regulation.

Recently, a novel 160-kDa substrate of Akt has been identified in 3T3L1 adipocytes as AS160, a protein containing a Rab GTPase-activating protein (GAP) domain (8). Phosphorylation of AS160 is required for the insulin-induced translocation of GLUT4 to the plasma membrane in 3T3L1 adipocytes (9). The expression of a dominant inhibitory mutant AS160 markedly reduces GLUT4 exocytosis, without altering endocytosis (10). We hypothesized that insulin action on AS160, the most proximal step identified thus far in the insulin-signaling cascade to glucose transport, is impaired in skeletal muscle from type 2 diabetic patients. Here we report that physiological hyperinsulinemia increases AS160 phosphorylation in human skeletal muscle. Moreover AS160 phosphorylation is impaired in type 2 diabetic patients. Our results further demonstrate that functional defects in insulin signaling are associated with impaired glucose transport in diabetic patients.

RESEARCH DESIGN AND METHODS

The study protocol was approved by the ethical committee of the Karolinska Institutet, 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 10 male type 2 diabetic patients. Glycemic control, as evaluated by HbA_{1c}, was moderate ($6.0 \pm 0.5\%$); the normal range for HbA_{1c} in our laboratory is <5.2%. The diabetic subjects were treated with a sulfonylurea ($n = 8$), insulin ($n = 1$), or a combination therapy of sulfonylurea and insulin ($n = 1$). The control group consisted of nine 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 48-h period before the study. The subjects reported to the laboratory after an overnight fast and, in the case of the type 2 diabetic patients, before the administration of diabetic medication.

A modification of the hyperinsulinemic clamp procedure was used in conjunction with an open-muscle biopsy to obtain vastus lateralis muscle (11). Muscle biopsies were obtained under local anesthesia (mepivacain chloride 5 mg/ml) 30 min after a glucose priming period (basal). A bolus injection of insulin was administered ($17.6 \ nmol \cdot kg^{-1} \cdot min^{-1}$) for 4 min, and hyperinsulinemia was maintained by a continuous insulin infusion ($5.5 \ nmol \cdot kg^{-1} \cdot min^{-1}$). A second biopsy (insulin-stimulated) was obtained 40 min after the onset of the insulin infusion. Serum insulin levels at the time of the second

TABLE 1
Clinical and metabolic characteristics of study participants

	Control	Type 2 diabetic
n	9	10
Age (years)	56 ± 2	54 ± 2
BMI (kg/cm²)	26.7 ± 0.8	27.0 ± 1.3
HbA_{1c} (%)	4.0 ± 0.2	$6.0 \pm 0.5*$
Glucose (mmol/l)	5.5 ± 0.1	10.1 ± 0.7†
Insulin (pmol/l)	45.4 ± 5.5	84.6 ± 16.0‡
Glucose during clamp (mmol/l)	5.8 ± 0.2	8.5 ± 1.1‡

Data are means $\pm$ SE. Glucose and insulin levels were measured after an overnight fast. $*P < 0.01$, $†P < 0.001$, and $‡P < 0.05$ vs. control.

muscle biopsy were ~600 pmol/l. Each biopsy was obtained from different muscle bundles from the same incision site using a Weil-Blakesley conchotome (12). Muscles were immediately frozen and stored in liquid nitrogen until analysis.

Tissue processing. Muscle biopsies (40–50 mg) were freeze-dried overnight and subsequently dissected under a microscope to remove visible blood, fat, and connective tissue. Muscles were homogenized in ice-cold homogenization buffer (90 μl/μg dry wt muscle; 20 mmol/l Tris [pH 7.8], 137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l $MgCl_2$, 1% Triton X-100, 10% [wt/vol] glycerol, 10 mmol/l NaF, 1 mmol/l EDTA, 5 mmol/l Na-pyrophosphate, 0.5 mmol/l Na_3VO_4, 1 μg/ml leupeptin, 0.2 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mmol/l dithiothreitol, 1 mmol/l benzamidine, and 1 μmol/l microcystin). Homogenates were rotated for 30 min at 4°C. Samples were subjected to centrifugation (12,000*g* for 15 min at 4°C); the protein concentration was determined in the supernatant using the BCA protein assay kit (Pierce, Rockford, IL). An aliquot of muscle homogenate (30 μg protein) was mixed with Laemmli buffer containing β-mercaptoethanol, heated at 60°C, and subjected to SDS-PAGE.

Immunoprecipitation. Aliquots of muscle lysate (300 μg protein) were immunoprecipitated overnight at 4°C with an antibody against the COOH-terminal 12 amino acids of mouse AS160 (PTNDKAKAGNKP). Human AS160 differs from mouse AS160 at four positions in this peptide (human sequence, NPNNKAKIGNKP), but the antibody reacts equally well with mouse and human AS160 (H. Sano and G.E.L., unpublished observations).

Samples were incubated with protein A sepharose beads for 2 h at 4°C and washed three times with homogenization buffer and four times with phosphate-buffered saline. The immunocomplex was resuspended in Laemmli buffer containing β-mercaptoethanol. Samples were heated at 95°C for 4 min and subjected to SDS-PAGE.

Western blot analysis. Proteins were separated by SDS-PAGE (7.5% resolving gel), transferred to nitrocellulose membranes, and blocked with Tris-buffered saline with 0.02% Tween (TBST) containing 5% milk for 2 h. Membranes were incubated overnight with anti−phospho-Akt (Ser473; catalog no. 9,271), anti−phospho-Akt (Thr308; catalog no. 9,275), anti−phospho-(Ser/Thr) Akt substrate (PAS; catalog no. 9,611), anti-Akt (catalog no. 9,272) (Cell Signaling Technology, Beverly, MA), or an antibody raised against the COOH-terminus of mouse AS160 (8). Membranes were washed in TBST and incubated with appropriate secondary horseradish peroxidase−conjugated antibodies (Bio-Rad, Richmond, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL plus; Amersham, Arlington Heights, IL) and quantified by densitometry using Molecular Analyst Software (Bio-Rad).

Statistical analysis. Data are presented as means $\pm$ SE. Student's unpaired *t* test was used to assess differences between control subjects and type 2 diabetic patients. Differences were considered significant at $P < 0.05$.

RESULTS

The healthy subjects and type 2 diabetic patients were matched for age and BMI (Table 1). As expected, the type 2 diabetic patients had significantly higher levels of HbA_{1c}, fasting glucose, and fasting plasma insulin.

We determined the effects of physiological hyperinsulinemia on phosphorylation of Akt substrates (Fig. 1). We detected several insulin-responsive proteins in crude lysates of skeletal muscle from control and type 2 diabetic
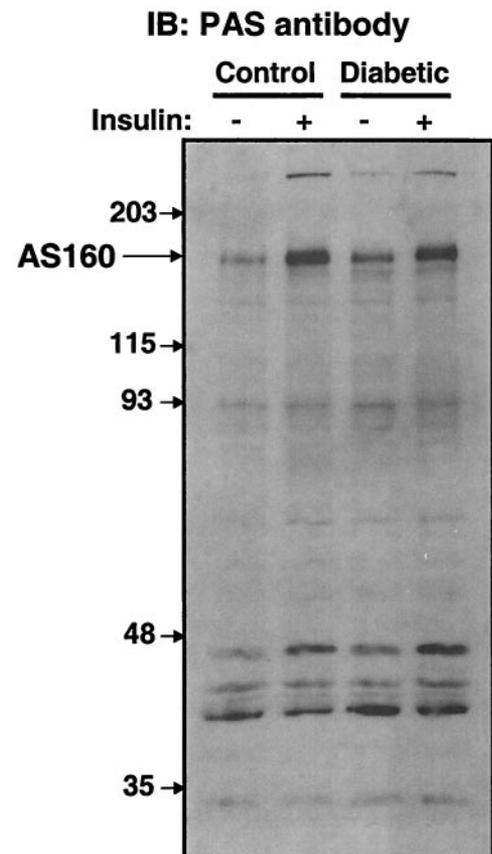

FIG. 1. Proteins detected by PAS antibody. Representative PAS immunoblot (IB) of basal and insulin-stimulated samples from one healthy subject and one type 2 diabetic patient. Multiple proteins were detected in the immunoblot with the PAS antibody. AS160 is indicated in the left margin of the blot.

subjects after immunoblot analysis with a PAS antibody. One of the most predominant immunoreactive bands was a phosphoprotein with a molecular weight of 160 kDa, presumably AS160. To identify this phosphoprotein as AS160, lysates of human skeletal muscle were immunoprecipitated overnight with an antibody raised against the COOH-terminus of mouse AS160. Muscle lysates, immunoprecipitated samples, and immunodepleted (post-immunoprecipitation) samples were immunoblotted with the PAS
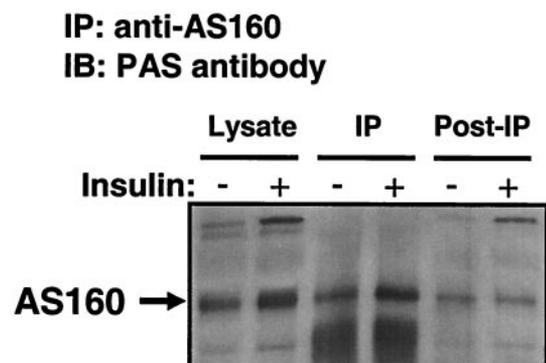

FIG. 2. Identification of AS160. Basal and insulin-stimulated human skeletal muscle lysate was immunoprecipitated with an antibody raised against the COOH-terminus of the mouse AS160. Crude lysate, the immunoprecipitate (IP), and the postimmunoprecipitated lysate (Post-IP) were run on a 7.5% SDS-PAGE. The membrane was immunoblotted with the PAS antibody.
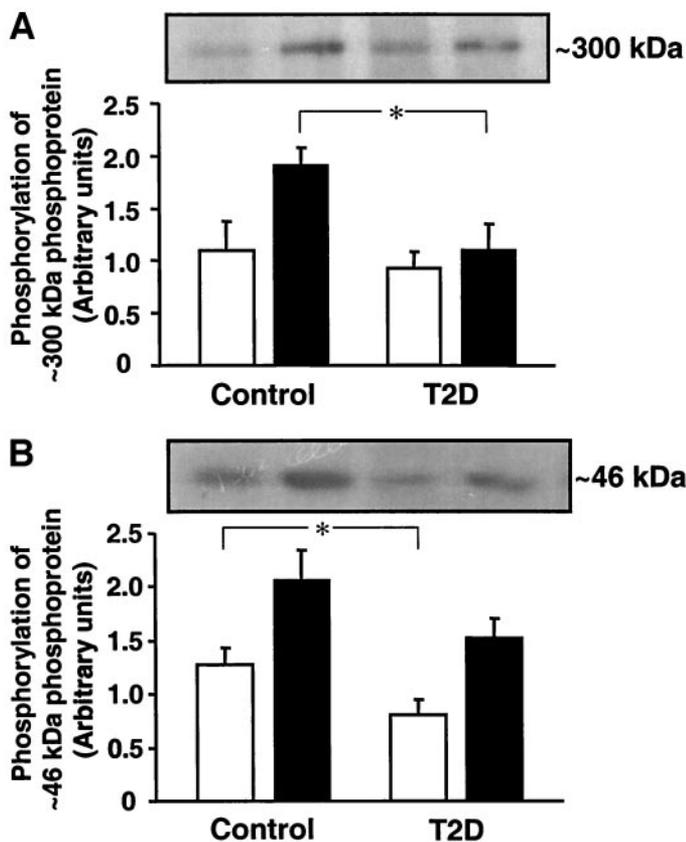
FIG. 3. Phosphorylation of high– (~300 kDa) and low– (~46 kDa) molecular weight phosphoproteins. Basal and insulin-stimulated phosphorylation of high– (*A*) and low–molecular weight (*B*) proteins in skeletal muscle from control subjects and type 2 diabetic patients are shown. A representative portion of an immunoblot with the PAS antibody is presented. Average densitometry values of basal samples on each immunoblot were multiplied to obtain the arbitrary level of 1, with the insulin values adjusted accordingly. □, basal condition; ■, insulin-stimulated condition. Data are means ± SE for basal (*n* = 6 control and *n* = 9 type 2 diabetes [T2D]) and insulin-stimulated (*n* = 8 control and *n* = 9 type 2 diabetes) conditions. *$P < 0.05$ control vs. type 2 diabetic subjects.

FIG. 4. Expression and phosphorylation of AS160. *A*: Representative immunoblot of AS160 protein expression probed with an antibody raised against the COOH-terminus of the mouse AS160. Duplicate samples of 30 μg protein from control (C) and type 2 diabetic (D) subjects were loaded in each lane. *B*: Basal and insulin-stimulated phosphorylation of AS160 in skeletal muscle from control subjects and type 2 diabetic patients (T2D). A representative portion of an immunoblot with the PAS antibody is shown. Average densitometry values of basal samples on each immunoblot were multiplied to obtain the arbitrary level of 1, with the insulin values adjusted accordingly. □, basal condition; ■, insulin-stimulated condition. Data are means ± SE for basal (*n* = 6 control and *n* = 9 type 2 diabetes) and insulin-stimulated (*n* = 8 control and *n* = 9 type 2 diabetes) conditions. *$P < 0.05$ control vs. type 2 diabetic subjects.

antibody (Fig. 2). This study revealed that the identity of the 160-kDa phosphoprotein was AS160, and it confirmed that physiological hyperinsulinemia specifically increases AS160 phosphorylation in human skeletal muscle.

In addition to AS160, two insulin-responsive phosphoproteins with molecular weights of ~300 and ~46 kDa, respectively, were identified in human skeletal muscle using the PAS antibody (Fig. 3). Basal phosphorylation of the high–molecular weight (~300 kDa) phosphoprotein was similar between control and type 2 diabetic subjects (Fig. 3*A*). Physiological hyperinsulinemia increased phosphorylation of the high–molecular weight protein 1.7-fold ($P < 0.05$) in control subjects, but had no significant effect in type 2 diabetic subjects. Basal phosphorylation of the low–molecular weight (~46 kDa) phosphoprotein was reduced 37% between control and type 2 diabetic subjects ($P < 0.05$) (Fig. 3*B*). Physiological hyperinsulinemia increased phosphorylation of the low–molecular weight protein 1.6-fold ($P < 0.05$) in control subjects, with a blunted response noted in type 2 diabetic patients.

We focused our characterization of Akt substrates on AS160, as this target has been linked to glucose transport. AS160 protein expression was similar between type 2 dia-
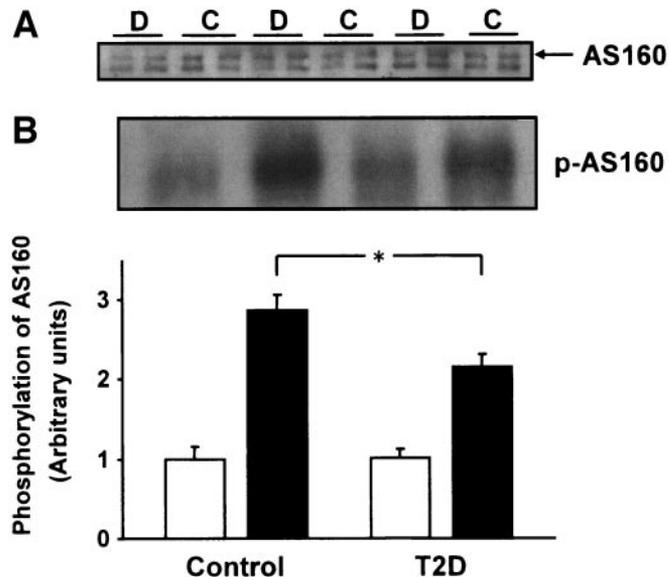
betic and healthy subjects (Fig. 4*A*). We next determined the effect of physiological hyperinsulinemia on AS160 phosphorylation (Fig. 4*B*). Basal AS160 phosphorylation was similar between control and type 2 diabetic subjects. Physiological hyperinsulinemia increased AS160 phosphorylation 2.9-fold ($P < 0.001$) and 2.1-fold ($P < 0.001$) in control and type 2 diabetic subjects, respectively. The insulin-stimulated AS160 phosphorylation increment over basal was reduced 39% ($P < 0.05$) in type 2 diabetic patients.

To determine whether reduced insulin action on AS160 was associated with impaired Akt protein expression or phosphorylation, we assessed Akt phosphorylation at Ser[473] and Thr[308]. Protein expression of Akt was similar between control and type 2 diabetic subjects (data not shown). Basal phosphorylation of Akt on Ser[473] and Thr[308] was similar between control and type 2 diabetic subjects. Physiological hyperinsulinemia increased Ser[473] phosphorylation of Akt by 4.1-fold ($P < 0.001$) and 3.1-fold ($P < 0.001$) in control and type 2 diabetic subjects, respectively (Fig. 5*A*). Although insulin action on Ser[473] phosphorylation tended to be reduced in type 2 diabetic patients, this difference was insignificant. Physiological hyperinsulinemia increased Thr[308] phosphorylation of Akt 3.1-fold ($P < 0.001$) and 2.0-fold ($P < 0.01$) in control and type 2 diabetic subjects, respectively (Fig. 5*B*). The insulin-stimulated increment in Thr[308] phosphorylation of Akt over basal was reduced 51% ($P < 0.01$) in type 2 diabetic patients.

DISCUSSION

Akt is a serine/threonine protein kinase that phosphorylates proteins with an RXRXXS/T motif. Using antibodies
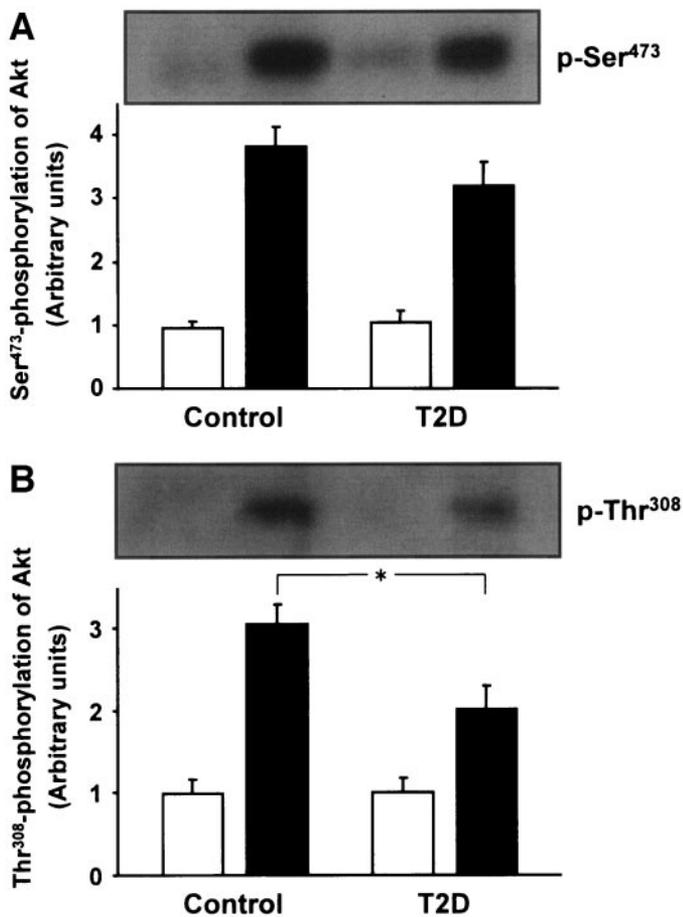
FIG. 5. Phosphorylation of Akt. Basal and insulin-stimulated Ser[473] (A) and Thr[308] (B) phosphorylation of Akt in skeletal muscle from control and type 2 diabetic (T2D) subjects. Representative portions of the immunoblots with the phosphospecific antibodies are shown. The weak bands of basal Thr[308] phosphorylation are not visible. Average densitometry values were calculated as described in Fig. 3. □, basal condition; ■, insulin-stimulated condition. Data are means ± SE for basal ($n = 6$ control and $n = 9$ type 2 diabetes) and insulin-stimulated ($n = 8$ control and $n = 9$ type 2 diabetes) conditions. *$P < 0.05$ control vs. type 2 diabetic subjects.

that specifically recognize the phosphorylated Akt substrate epitope, a novel 160-kDa Akt substrate (AS160) was identified in 3T3L1 adipocytes (8). Insulin increases AS160 phosphorylation at five residues within RXRXXS/T motifs (9). In adipocytes transfected with a mutated form of AS160, where several of the phosphorylation sites are mutated to alanine, translocation of GLUT4 from intracellular compartments to the plasma membrane is markedly impaired (9,10). These studies (9,10) revealed that AS160 is the most proximal component of the insulin-signaling cascade linked to glucose transport.

The role of AS160 in insulin signal transduction is not limited to adipocytes. mRNA of AS160, also designated as KIAA0603 (TBC-1 domain family member 4), is highly expressed in human heart and skeletal muscle tissue compared with other tissues (13). Furthermore, in rat epitrochlearis skeletal muscle, a 160-kDa protein, presumably AS160, is phosphorylated in a dosage-dependent manner by insulin during in vitro incubations (14,15). AS160 is likely to play an important role in insulin action on GLUT4 translocation and glucose transport in skeletal muscle. Here we have provided evidence that physiologi-

cal hyperinsulinemia increases AS160 phosphorylation in human skeletal muscle. Moreover, we have reported that insulin action on AS160 is impaired in type 2 diabetic patients.

In addition to AS160, three novel Akt substrates (molecular weights 250, 105, and 47 kDa) have been identified in 3T3L1 adipocytes (16). Other novel Akt substrates have also been recently identified (rev. in 17). In rat skeletal muscle, in vitro contraction is associated with an insulin-independent increase in the phosphorylation of AS160, pp180, and pp250 (14). We also detected several immunoreactive proteins in human muscle in addition to AS160 using the PAS antibody, two of which were clearly phosphorylated in response to insulin infusion (~300 kDa and ~46 kDa). We hypothesized that the protein with the higher molecular weight was mammalian target of rapamycin (mTOR), based on the evidence that phosphorylation of Ser[2448] is controlled by Akt (18). Furthermore, mTOR Ser[2448] and the surrounding sequence share homology with the consensus motif RXRXXS/T that is recognized by the PAS antibody (19). To determine whether the high−molecular weight protein was mTOR, we stripped the PAS immunoblot (Fig. 1) and reprobed with anti-mTOR. Immunoblot analysis of mTOR protein expression in human skeletal muscle (data not shown) indicated that the high−molecular weight protein did not correspond to mTOR. A similar approach was taken to reveal the nature of the protein with the lower molecular weight detected at ~46 kDa. Because glycogen synthase kinase 3α (GSK-3α) is a 46-kDa Akt substrate, it was a plausible candidate for the low−molecular weight protein detected (Fig. 1). Reprobing the PAS immunoblot with anti−GSK-3α/β revealed the lower molecular weight protein co-migrated with GKS-3α. Although the exact nature of the low− and high−molecular weight phosphoproteins detected in the human muscle is unknown, physiological hyperinsulinemia increased phosphorylation of these proteins, with blunted responses observed in type 2 diabetic subjects.

AS160 protein expression was unaltered between type 2 diabetic and healthy subjects. Thus, the reduction in insulin action on AS160 phosphorylation in skeletal muscle from type 2 diabetic patients may be explained by impaired early signaling events at the level of IRS-1/PI 3-kinase/Akt. Although several groups have reported insulin-signaling defects at the level of IRS-1/PI 3-kinase in skeletal muscle from type 2 diabetic patients, results concerning Akt have been inconclusive (7). After insulin stimulation, the generation of PI 3,4,5-trisphosphate by PI 3-kinase is necessary for Akt to relocalize to the cell membrane via interaction of the NH2-terminal pleckstrin homology domain. Akt is activated by two phosphorylation steps (rev. in 20). First, Akt is phosphorylated at Ser[473] in a hydrophobic motif at the COOH-terminal tail. The identity of the kinase responsible for the phosphorylation at Ser[473] has been debated, but results from a recent study suggest that DNA-dependent protein kinase is a candidate (21). Akt is then phosphorylated at Thr[308] in the catalytic domain by 3-phosphoinositide–dependent kinase 1 to achieve full activation of the kinase. Here we report that phosphorylation of Ser[473] was similar between healthy and type 2 diabetic subjects. This finding is consistent with those of previous studies, whereby Akt phos-

phorylation/activity has been reported to be unaltered in skeletal muscle from type 2 diabetic subjects in response to physiological hyperinsulinemia (3,22). However, we also report that Akt phosphorylation at Thr^{308} was impaired in this type 2 diabetic cohort. The discrepancy between the reported unchanged Akt activity (3,22) and the reduced Thr^{308} phosphorylation observed in the present study is unclear. However, isoform-specific impairments in insulin action of Akt- 2 and -3, but not -1, have been observed in skeletal muscle from morbidly obese insulin-resistant subjects (23). Thus, Akt isoform-specific defects could potentially account for the differences in insulin action on Akt activity and phosphorylation as assessed in our diabetic cohort. The impairment in insulin action Thr^{308} phosphorylation may also be a consequence of the altered metabolic milieu, as our diabetic patients were studied under hyperglycemic conditions. Consistent with this hypothesis, an earlier study has revealed that insulin-induced Thr^{308} phosphorylation was unchanged between control and type 2 diabetic patients after an overnight normalization of glycemia (24). The reduction in Thr^{308} phosphorylation of Akt in our type 2 diabetic cohort coincides with reduced phosphorylation of the downstream Akt substrate AS160.

AS160 links insulin signaling via PI 3-kinase/Akt to GLUT4 translocation. We have previously reported that GLUT4 translocation is impaired in skeletal muscle from type 2 diabetic patients (25,26). An hypothesis for the involvement of AS160 in mediating translocation of GLUT4-containing vesicles to the plasma membrane is based on evidence from experiments using different mutated forms of AS160 (9,10). AS160 contains a GAP domain for Rabs, which are small G proteins required for membrane trafficking (9). During unstimulated conditions, Rabs are maintained in an inactive guanosine diphosphate form by the active GAP of AS160. Insulin-stimulated phosphorylation of AS160 signals GLUT4 translocation through inactivation of the Rab GAP function (9). Although the exact nature of the Rab target for AS160 is unknown, Rab-4 and -11 have been linked to GLUT4 traffic in insulin-sensitive tissues. Rab-4 has been shown to coprecipitate with GLUT4-containing vesicles in intracellular membrane fractions in rat skeletal muscle (27,28) and upon insulin stimulation, the abundance of Rab-4 was decreased in intracellular membrane compartments and undetected at the plasma membrane (28). It has been suggested that Rab-11 is involved in the endosomal recycling, sorting, and exocytotic movement of GLUT4 in rat cardiac muscle (29). Thus, AS160 may constitute a convergence between insulin signaling and vesicular trafficking.

In summary, insulin phosphorylates several Akt substrates in human skeletal muscle. We have provided evidence that physiological hyperinsulinemia leads to a robust phosphorylation of AS160 in human skeletal muscle. Moreover, we have revealed that in type 2 diabetic patients, skeletal muscle protein expression of AS160 is unaltered, but insulin-induced phosphorylation is attenuated. Impaired insulin action on AS160 was associated with reduced Akt Thr^{308} phosphorylation, suggesting that these two events are linked. Given the evidence that AS160 is required for insulin-mediated GLUT4 translocation in 3T3L1 adipocytes (9,10), our data suggest aberrant insulin signaling to AS160 contributes to defects in GLUT4 translocation and glucose uptake in skeletal muscle in insulin-resistant type 2 diabetic patients.

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