Impaired expression and insulin-stimulated phosphorylation of Akt-2 in muscle of obese patients with atypical diabetes

Aidar R. Gosmanov,1 Guillermo E. Umpierrez,2 Ana H. Karabell,3 Ruben Cuervo,2 and Donald B. Thomason1

Departments of 1Physiology and 2Medicine, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38163; and 3Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322

Submitted 27 October 2003; accepted in final form 27 February 2004

Gosmanov, Aidar R., Guillermo E. Umpierrez, Ana H. Karabell, Ruben Cuervo, and Donald B. Thomason. Impaired expression and insulin-stimulated phosphorylation of Akt-2 in muscle of obese patients with atypical diabetes. Am J Physiol Endocrinol Metab 287: E8–E15, 2004. First published March 9, 2004; 10.1152/ajpendo.00485.2003.—Although a pharmacological dose of insulin produces a dramatic increase in phosphorylation and activity of Akt isoforms 1 and 2 in mammalian skeletal muscle, few studies have examined the effect of physiological concentrations of insulin on the phosphorylation of Akt-1 and -2 in normal and diabetic tissue. This study examined the patterns of insulin-stimulated Akt isoform phosphorylation and protein expression in muscle biopsies obtained from obese patients with atypical diabetes immediately after a hyperglycemic crisis and again after near-normoglycemic remission. In obese patients with new-onset diabetes mellitus presenting with hyperglycemic crisis (plasma glucose 30.5 ± 4.8 mM), in vitro stimulation of vastus lateralis muscle biopsies with 100 μU/ml (0.6 nM) insulin increased insulin receptor phosphorylation threefold and Akt-1 phosphorylation on Ser473 twofold, whereas Akt-2 phosphorylation was not stimulated. After 10-wk intensive insulin therapy that led to near-normoglycemic remission and discontinuation of insulin therapy, both Akt-2 expression and insulin-stimulated Akt-2 Ser474 phosphorylation doubled. Hyperglycemic crisis did not affect insulin-stimulated threonine phosphorylation of either Akt-1 or Akt-2. The decreased Akt-2 expression at presentation was accompanied by reduced GLUT4 protein expression and increased expression of enzymes counterregulatory to insulin action. Thus a physiological concentration of insulin stimulated Akt-1 and Akt-2 phosphorylation in human skeletal muscle in the absence of hyperglycemia, but Akt-2 expression and stimulation appeared to be impaired in muscle of obese patients with atypical diabetes presenting with severe hyperglycemia.

hyperglycemia; Akt-1; insulin receptor; insulin receptor substrate; glucose transporter 4

In skeletal muscle, the acute action of insulin results in stimulation of glucose uptake and glycogen synthesis (4, 11, 18, 27, 32, 40). Insulin exerts its biological effects by sequential activation of a cascade of signaling molecules (22, 45). Insulin binds to its receptor, which in turn leads to receptor autophosphorylation and activation of several docking proteins containing a phosphotyrosine-binding domain, including insulin receptor substrates (IRS) 1–4. In mammalian skeletal muscle cells, it has been shown that IRS-1/2 proteins are indispensible for the insulin effects on glucose utilization (39, 48). Tyrosine phosphorylation of IRS-1/2 allows interaction with and activation of phosphatidylinositol 3-kinase (PI3K). Phosphatidylinositol 3,4,5-triphosphate (PIP3) production subsequently recruits the serine-threonine kinase Akt (protein kinase B) and its activating kinase 3-phosphoinositide-dependent protein kinase-1 (PDK-1) to the membrane to initiate activation of Akt (7). Full activation of Akt occurs only when Akt is dually phosphorylated on a threonine residue by PDK-1 and a serine residue by a putative PDK-2 (or kinase complex) that has yet to be fully characterized (2, 17, 42). Akt activation is required and may be sufficient for stimulation of glucose transport and glycogen accumulation in skeletal muscle cells (2, 4, 14, 47). In mammalian tissues, there are three Akt isoforms (Akt-1, -2, and -3), encoded by distinct genes. Insulin action in muscle predominantly involves Akt-1 and Akt-2 stimulation (25, 43, 46), and Akt-2 knockout mice have impaired glucose homeostasis, a diabetes-like syndrome (4, 5).

It is known that non-insulin-dependent diabetes mellitus (type 2 diabetes mellitus) is accompanied by a diminished ability of insulin to activate Akt (3, 29, 37), whereas insulin-sensitizing drugs improve insulin-stimulated Akt activity (24, 34). Hyperglycemia is now recognized as a deleterious factor that can lead to insulin resistance independent of genetic background (9, 28, 35, 45, 51). In the case of skeletal muscle, there is evidence that hyperglycemia by itself may decrease Akt activation (12, 30). Furthermore, experimental data demonstrate that hyperglycemia can cause alterations in the profile of cell protein expression such that insulin-counterregulatory mechanisms could prevail (9). Therefore, the correlative ties between muscle Akt signaling and patterns of protein expression during hyperglycemia are of great interest. One model in which to study the effects of hyperglycemia is a unique form of type 2 diabetes called atypical type 2 diabetes. This form of diabetes is a common clinical presentation involving ∼50% of adult African Americans (and, increasingly, Hispanic and Native Americans) with obesity and newly diagnosed diabetes who present with ketosis and/or severe hyperglycemia. Although these patients are severely insulin resistant and insulinopenic on presentation, with intensive insulin therapy the majority of patients achieve near-normoglycemic remission within ∼10–12 wk, become more insulin sensitive, and do not
require exogenous insulin (1, 33, 44). Therefore, an analysis of muscle Akt activation in these patients at presentation and remission provides an ideal opportunity to examine the association of hyperglycemia with Akt isoform activation.

In this study, we investigated the effect of insulin at physiological concentrations on Akt-1 and Akt-2 phosphorylation in muscle biopsies obtained from obese patients with atypical diabetes immediately following a hyperglycemic crisis and again following near-normoglycemic remission. Our experiments demonstrated that hyperglycemia is associated with a decrease in muscle Akt-2 expression and insulin-stimulated phosphorylation on the serine residue, without affecting threonine phosphorylation. Furthermore, data from antibody arrays comparing protein expression at patient presentation relative to near-normoglycemic remission indicated a significant correlation between Akt-2 expression and Ser\textsuperscript{473} phosphorylation with a number of proteins, including several that may counteract insulin action in mammalian tissues.

**MATERIALS AND METHODS**

**Materials.** Insulin was purchased from Sigma Chemical (St. Louis, MO). Phosphospecific antibodies to Akt on Thr\textsuperscript{116/118} and on Ser\textsuperscript{473}, and anti-total-Akt antibody were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to phosphotyrosine (PY-99, sc-7020), insulin receptor β-subunit (sc-711), IRS-1 (sc-7200), IRS-2 (sc-8299), Akt-1 (sc-1618), Akt-2 (sc-5270), GLUT4 (sc-7938), extracellular signal-regulated kinase-1 (ERK1; sc-94-G), ERK2 (sc-153), and p38 (sc-535) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody Microarray 380 (catalog no. K1847–1) was purchased from BD Biosciences (Palo Alto, CA). All other chemicals were from Sigma Chemical.

**Human subjects and muscle biopsy.** The study group included four obese (2 males and 2 females) African American patients with new onset of diabetes mellitus who presented in hospital with severe hyperglycemia (glucose \textgreater 400 mM). Obesity was defined as body mass index (BMI) \textgreater 28 kg/m\textsuperscript{2}. The study protocol was approved by the Institutional Review Board of University of Tennessee Health Science Center and performed in the General Clinical Research Center. Written informed consent was obtained from all subjects. The Institutional Review Board of University of Tennessee Health Science Center and performed in the General Clinical Research Center. Written informed consent was obtained from all subjects.

**Muscle incubation and processing.** Each muscle sample was attached to a glass wand at its resting length (no load) with a silk suture for rapid transfer among solutions. The muscles were preincubated for 15 min at 30°C in oxygenated Krebs-Ringer solution. For insulin stimulation, after preincubation the muscle was taken directly to Krebs-Ringer solution containing 100 μU/ml insulin for 5 min. The muscle designated as the treatment control was similarly incubated but without insulin. Thereafter, the muscle was quickly washed, blotted, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis. The muscle was homogenized in solubilization buffer at 4°C, as previously described (13), and centrifuged at 15,000 × g for 10 min. The protein concentration of the supernatant was measured in triplicate with the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL).

**Immunoprecipitation and Western blotting.** To measure tyrosine phosphorylation of the insulin receptor (InsR), 1,000 μg of protein from muscle lysates were first incubated with anti-InsR antibody for 2 h at 4°C and then subjected to overnight immunoprecipitation with protein A-agarose beads. The immune complexes were washed three times with lysis buffer, mixed with SDS denaturing buffer, warmed to 95°C for 5 min, electrophoresed on a 6% SDS-PAGE gel, and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Tyrosine-phosphorylated proteins were visualized by incubation of membranes with phosphotyrosine PY-99 and horseradish peroxidase (HRP)-conjugated anti-mouse antibodies in blocking buffer (1.5 mM Na\textsubscript{3}PO\textsubscript{4}, 8 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.15 M NaCl, 0.3% Triton X-100, pH 7.4) containing 1% BSA at room temperature, followed by chemiluminescent exposure of X-ray film (ECL Plus). By reprobing the membranes with anti-InsR, the antibody measured the total amount of corresponding protein. Bands were quantitated by video densitometry. Protein phosphorylation was calculated as the ratio of phosphorylated (phospho) to total protein expression, normalized to a mean basal value (no insulin stimulation) of 1.0.

To determine phosphorylation and abundance of Akt in whole muscle lysates, equal amounts of protein were resolved by 10% SDS-PAGE, transferred to PVDF membranes, and blocked overnight in 3% BSA blocking buffer. The membranes were incubated at room temperature with anti-phospho-Akt-Thr\textsuperscript{308} antibody and HRP-conjugated anti-rabbit IgG, followed by chemiluminescent exposure of X-ray film. The same membranes were then stripped two times to determine total Akt expression and phospho-Akt-Ser\textsuperscript{473}, phosphor- ylation by use of anti-total-Akt and anti-phospho-Akt-Ser\textsuperscript{473} antibody, respectively. The antibodies used in these experiments recognized the phosphorylation status and expression of all Akt isoforms. Bands were quantitated by video densitometry. Protein phosphorylation was calculated as the ratio of phosphorylated to total protein expression, normalized to a mean basal value of 1.0. GLUT4 abundance in whole muscle lysate was similarly determined by Western blot analysis.

**To measure Akt-1 and Akt-2 phosphorylation, muscle lysates were immunoprecipitated with Akt-1 or Akt-2 isoform-specific antibodies, respectively, for 2 h at 4°C, followed by overnight incubation with protein A-agarose beads. After being washed, immune complexes were resolved on 10% SDS-PAGE and electroblotted onto PVDF membranes. Akt-1 and Akt-2 phosphorylation on threonine (Thr\textsuperscript{308} for Akt-1 and Thr\textsuperscript{306} for Akt-2) or serine (Ser\textsuperscript{473} for Akt-1 and Ser\textsuperscript{474} for Akt-2) was determined, as described above. Specificity of the Akt-1 and the Akt-2 immunoprecipitation was determined by testing for cross-reactivity of the immunoprecipitates. No cross-reactivity was detected.

**Antibody array analysis.** Antibody arrays from BD Biosciences (lot no. 2090032) were prepared according to product instructions. Briefly, 1,000-μg aliquots of protein lysate from muscle biopsies taken at presentation and at remission were each labeled with Cy3 and Cy5 dyes (Amersham Biosciences, Piscataway, NJ). Unbound dye was removed from the samples by filtration through PD-10 columns (Amersham Biosciences). Labeled efficiency was estimated from dye absorbance and was typically 2.5–3 molecules of dye per protein molecule. Cy3 dye-labeled protein (100 μg) from the presentation sample was mixed with 100 μg of Cy5 dye-labeled protein from the
remission sample. Similarly, 100 μg of Cy5 dye-labeled protein from the presentation sample was mixed with 100 μg of Cy3 dye-labeled protein from the remission sample. Each of these two mixes was incubated separately with antibody microarray slides for 30 min and washed. The slides were dried and analyzed immediately in a ScanArray 5000XL confocal laser scanner. To correct for differences in dye labeling efficiency, we calculated for each sample an internally normalized ratio [the square root of ratio of the Cy5 (presentation)/Cy3 (remission) fluorescence from one slide and Cy5 (remission)/Cy3 (presentation) fluorescence from the other slide] for each of the duplicate antibody-antigen spots on each array. The internally normalized ratio is the expression of the antigen at presentation relative to remission. Data are deposited in the Gene Expression Omnibus repository at http://www.ncbi.nlm.nih.gov/geo, series record GSE634.

Statistics. Comparisons within and among groups for insulin-stimulated phosphorylation and expression were made by analysis of variance. Differences were considered significant if P < 0.05. Data are reported as means ± SE. Standard errors that were too small to be distinguished from the data points were not plotted. For differences that were not significant, the power of these tests was typically >0.85. Overrepresentation of molecular function gene ontologies was calculated using the EASE program (16).

RESULTS

Insulin-stimulated phosphorylation of total Akt, Akt-1, and Akt-2 in muscle biopsies of patients with severe hyperglycemia. The clinical characteristics and selected metabolic parameters of study participants are shown in Table 1. Middle aged, obese (BMI > 28 kg/m²) subjects presenting with severe hyperglycemia were included in the study (mean fasting plasma glucose 30.5 ± 4.8 mM or 550 ± 86.5 mg/dl). After ~10 wk of treatment with subcutaneous insulin and ≥1 wk of discontinuation of insulin therapy, fasting blood glucose significantly decreased to near-normoglycemic values. BMI and plasma insulin did not change during treatment.

Muscle biopsies obtained at presentation with severe hyperglycemia and following near-normoglycemic remission were incubated in the absence or presence of 100 μU/ml insulin. To ensure that the insulin effect on Akt signaling was associated with canonical activation of insulin receptor tyrosine phosphorylation, muscle lysates of three patients were immunoprecipitated with InsR antibody. We found a similar threefold stimulation of InsR tyrosine phosphorylation by insulin at presentation and near-normoglycemic remission (Fig. 1). Regardless of glycemic status, insulin increased Thr308/309 phosphorylation of total Akt 2.2- to 2.5-fold (Fig. 1). Consistent with these results, insulin increased Akt-1 and Akt-2 phosphorylation on Thr308/309 at presentation and remission (Fig. 2). In contrast, insulin did not stimulate Ser473 phosphorylation of total Akt at presentation, although insulin-stimulated phosphorylation tended to be greater at remission (Fig. 2). It is possible to compare the presentation values with the remission values only for a particular residue of an individual isoform, because different combinations of antibodies (which have different avidities of their immune complexes) were used to detect the Akt isoforms and their phosphorylated residues. Assessing the contribution of the Akt isoforms to total Akt phosphorylation on serine, we found that Akt-1 phosphorylation on Ser^73 was stimulated by insulin during the hyperglycemic period and remission. In contrast, insulin was not able to increase Akt-2 phosphorylation on Ser^474 at presentation with hyperglycemia. However, in near-normoglycemic remission, insulin caused a marked elevation of Akt-2 phosphorylation (Fig. 2). In addition, Akt expression was 69% higher at remission (P < 0.05; Fig. 2). This elevation in Akt expression at remission appeared to be largely due to a 94% increase in Akt-2 abundance (Fig. 2). Because of the improved Akt expression at remission, we asked whether there was a pattern of signal transduction protein expression associated with the severe hyperglycemia.

Pattern of protein expression in muscle biopsies of patients with severe hyperglycemia. We used antibody arrays to measure the expression of 380 proteins in muscle biopsies taken at presentation and remission. For selected proteins (InsR, GLUT4, ERK1/2, p38 MAPK), simultaneous Western blot analysis was used to confirm the antibody array data and to provide additional data; there was significant correspondence between data obtained by Western blot and antibody array (P < 0.001, contingency coefficient = 0.775). The expression of each protein at presentation was normalized to its expression

Table 1. Clinical and metabolic characteristics of patients at presentation and after near-normoglycemic remission

<table>
<thead>
<tr>
<th></th>
<th>Presentation</th>
<th>Remission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>42.3±4.9</td>
<td>42.5±4.9</td>
</tr>
<tr>
<td>Weight, lb</td>
<td>216.0±15.9</td>
<td>218.8±18.2</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>36.0±2.8</td>
<td>36.4±3.0</td>
</tr>
<tr>
<td>Fasting plasma glucose, mM</td>
<td>30.5±4.8</td>
<td>6.9±1.1*</td>
</tr>
<tr>
<td>Fasting plasma insulin, μU/ml</td>
<td>31.1±20.4</td>
<td>22.0±4.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 patients. *P < 0.05 vs. presentation.

Fig. 1. A physiological concentration of insulin stimulated tyrosine phosphorylation of the insulin receptor (InsR) to a similar extent in human vastus lateralis muscle during a hyperglycemic episode and after near-normoglycemic remission. Human muscle biopsies (~100 mg) obtained during severe hyperglycemia (presentation) and after near-normoglycemic remission were each divided into 2 portions and incubated in the presence or absence of 100 μU/ml insulin for 10 min. Protein (1 mg) from each muscle lysate was immunoprecipitated (IP) with an anti-InsR antibody and subjected to Western blot (WB) analysis with an anti-phosphotyrosine antibody. The immunoblots were then stripped and reprobed with an anti-InsR antibody. Representative blots are shown. The intensity of each band was quantified and the phospho/total ratio was calculated for each sample. The phospho/total ratio data are normalized to the mean basal level of phosphorylation at presentation (taken as 1.0). Data were obtained from muscle samples of 3 different patients; presentation and remission samples were analyzed simultaneously on a separate blot for each patient. Values are means ± SE. *P < 0.05 vs. basal phosphorylation.
significant number of proteins was correlated among patients, with a distinct trend toward decreased expression at presentation (shown for 2 patients in Fig. 3). A subset of 64 proteins was identified whose expression was decreased at presentation by \( \geq 10\% \) and whose range of relative expression (presentation/remission) among the patients was \( <15\% \) (Fig. 3). Among these proteins that had decreased expression at presentation, molecular function gene ontologies comprising intracellular signaling were overrepresented (on the basis of their expected frequency of occurrence within the entire array and their observed occurrence within the subset of proteins; Fig. 4). The decreased expression of specific proteins at presentation was apparently not a generalized catabolism of muscle protein,

at remission (a value of \( \leq 1 \) represents decreased expression at presentation). There were no differences between muscle samples obtained at presentation and remission in the expression of InsR, IRS-1, and PI3K (Fig. 3). GLUT4 abundance trended lower \( (P = 0.06) \) at presentation (Fig. 3) but not to the same extent as the decreased Akt-2 expression. The expression of a
significance, only 19 proteins would have been expected to be necessary for many of insulin signaling (25, 43, 46). Akt isoforms are differentially activated by insulin (25, 43, 46). An emerging body of evidence demonstrates that Akt isoforms are differentially activated by insulin (25, 43, 46).

Fig. 4. Several molecular function gene ontologies were overrepresented within the subset of 64 proteins whose expression was decreased at presentation with severe hyperglycemia. The observed occurrence within the subset of proteins was significantly greater than the expected frequency on the basis of the representation of the ontologies within the entire array. Molecular function ontologies are listed in order of their EASE score (Fisher exact P corrected for sample size).

because the expression of many proteins was unchanged (as exemplified by the expression of InsR, IRS-1, and P3K) or increased (Fig. 3); the protein mass profiles (determined by surface-enhanced laser desorption/ionization, or SELDI) were also not different between presentation and remission (not shown). To determine which proteins were associated with the decreased Akt-2 expression and activation at presentation, we calculated the correlation coefficient for the relative expression of specific proteins (presentation/remission) vs. relative Akt-2 expression or insulin-stimulated Akt-2 Ser474 phosphorylation (presentation/remission) (Table 2). Consistent with the over-representation of signal transduction functional ontologies among the proteins whose expression was decreased at presentation, there was significant correlation between Akt-2 expression and insulin-stimulated Ser474 phosphorylation at presentation and the expression of many signal transduction proteins associated with insulin signaling (P < 0.05). At this level of significance, only 19 proteins would have been expected to correlate with Akt-2 expression or insulin-stimulated Ser474 phosphorylation through random variation.

DISCUSSION

A hallmark of insulin action in skeletal muscle is stimulation of Akt phosphorylation and activity. Akt activation appears to be necessary for many of insulin’s biological effects (reviewed in Refs. 2, 15, 22, 31). Three Akt proteins, Akt-1, -2, and -3, have been identified in mammalian cells, including muscle cells (46). An emerging body of evidence demonstrates that Akt isoforms are differentially activated by insulin (25, 43, 46).

Furthermore, it has been shown that the absence of Akt-2 in skeletal muscle of mice leads to impaired insulin-mediated glucose uptake, thereby contributing to the development of a diabetes-like syndrome (4, 5). Recently, Jiang et al. (21) demonstrated that partially depleting Akt-2 in 3T3-L1 adipocytes with small interfering RNA significantly diminishes insulin-stimulated hexose transport and glycogen synthase kinase-3 phosphorylation; almost complete Akt-1 depletion had much less effect (21), indicating a pivotal role for Akt-2 in insulin responsiveness. The data presented here show that both Akt-1 and Akt-2 can be fully phosphorylated by a physiological dose of insulin in the muscle of normoglycemic humans. However, diminished Akt-2 expression and insulin-stimulated phosphorylation of Akt-2 on Ser474 is associated with hyperglycemia in diabetic humans.

It is well documented that a physiological dose of insulin (100 μU/ml, or 0.6 nM) stimulates glucose uptake in human and rodent skeletal muscle ex vivo (4, 11, 18, 27, 32, 40). We verified that in human muscle this concentration of insulin activated the proximal element of insulin signaling, InsR (Fig. 1). The recruitment and subsequent activation of P3K by IRS-1 and -2 result in generation of PIP3, which, in turn, leads to translocation of Akt from the cytosol to the plasma membrane and activation of PDK-1 (7). Akt is fully active only when it is dually phosphorylated on the Thr308/309 residue by PDK-1 and on Ser473/474. The kinase responsible for Ser473/474 phosphorylation is still elusive. Although there is evidence arguing for either PDK-1 or autophosphorylation (2), recent data indicate the existence of a distinct PDK-2 activity that appears to be associated with the cytoskeleton and to involve the integrin-linked kinase (17, 42). In previous studies, we found that 100 μU/ml insulin is effective in Akt phosphorylation in rat skeletal muscle (13). However, Akt phosphorylation by physiological insulin concentration was very different in muscle from diabetic patients with severe hyperglycemia (Fig. 2).

Hyperglycemia may contribute to the development and progression of diabetes mellitus by directly diminishing the insulin responsiveness of skeletal muscle (45, 51). Indeed, correction of hyperglycemia in diabetic rodents and humans improves skeletal muscle insulin sensitivity in vivo and in vitro (10, 38, 49, 50). Diminished Akt activation has been proposed as a probable mechanism for hyperglycemia-induced insulin resistance in skeletal muscle (29, 41, 51). Hyperglycemia in concentrations up to 30 mM can directly decrease insulin-induced Akt phosphorylation on Ser473/474 in rat skeletal muscle (30, 35, 37). Previously, there was no evidence for an effect of hyperglycemia on Akt isoform phosphorylation in muscle of patients with diabetes mellitus. Atypical type 2 diabetes is an ideal population to test for an effect of hyperglycemia on muscle Akt activation, because the majority of newly diagnosed patients achieve near-normoglycemic remission (1, 33, 44). Because of their clinical course, the presence of pancreatic insulin reserve at presentation and remission, and the absence of autoimmune markers, such patients are recognized as having an atypical type 2 diabetes. Importantly, in these patients hyperglycemic crisis is associated with severe insulin resistance, whereas restoration of blood glucose level increases peripheral insulin sensitivity. Here, we show that overt hyperglycemia is associated with decreased stimulation of Akt serine phosphorylation by a physiological concentration of insulin without changes in Akt threonine phosphorylation (Fig. 2). Intriguingly, only Akt-2-Ser474 phosphorylation was impaired by the hyperglycemia, whereas physiological insulin was able to stimulate Akt-1 phosphorylation regardless of the patient’s glycemic status (Fig. 2). Although the data were obtained from four patients, the latter result is consistent with the reports that Akt-1 activation by a physiological concentration of insulin is...
Table 2. Relative expression of specific proteins in muscle samples (presentation/remission) correlated with relative Akt-2 expression and insulin-stimulated Akt-2 Ser1474 phosphorylation (presentation/remission)

<table>
<thead>
<tr>
<th>Protein</th>
<th>r</th>
<th>Positive</th>
<th>r</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin filament-associated protein</td>
<td>0.997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP kinase-β</td>
<td>0.959</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA fragment factor 45 (ICAD)</td>
<td>0.999</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dual homolog AI</td>
<td>0.999</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DYRK1</td>
<td>0.997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoglin</td>
<td>0.967</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK1</td>
<td>0.994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP1K</td>
<td>0.994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK1</td>
<td>0.960</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK5</td>
<td>0.994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin II</td>
<td>0.979</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin II</td>
<td>0.990</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>0.981</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokine-inducible kinase (Fnk)</td>
<td>0.993</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIM domain binding 3</td>
<td>0.994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKC-ε</td>
<td>0.994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKC-θ</td>
<td>0.994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polo-like kinase 1</td>
<td>0.994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcription elongation factor</td>
<td>0.956</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B polypeptide 1 (SII p15)</td>
<td>0.953</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only significant correlations are shown. Significance of the correlation was tested at P < 0.05 for 380 proteins.

not impaired in the muscle of mildly hyperglycemic diabetic patients and diabetic rats (29, 40). In both of these studies, insulin-stimulated glucose transport by muscle was reduced despite normal Akt-1 activation (29, 40), in accord with the data from Akt knockout and small interfering RNA studies supporting a pivotal role of Akt-2 over Akt-1 for glucose uptake and disposal (4, 5, 21). Further support for a differential effect of hyperglycemia on Akt-2 and Akt-1 comes from the analysis of Akt isoform expression (Fig. 2). We detected 70% greater total Akt expression in muscle of patients in near-normoglycemic remission compared with the hyperglycemic period. Increased Akt expression was associated with an almost twofold increase in Akt-2 abundance but only a slight elevation in Akt-1 abundance. Increased total Akt and Akt-2 abundance at remission, combined with no change or an increase in insulin-stimulated phosphorylation (for total Akt and Akt-2, respectively), would result in a greater amount of phosphorylated protein. By mass action, this may result in a greater magnitude of insulin-stimulated signal. However, in the case of Akt-2, basal phosphorylation (i.e., not insulin stimulated) is diminished at remission (Fig. 2), counteracting the gain in the amount of the phosphorylated protein and raising the possibility of enhanced sensitivity to phosphorylated Akt-2. Together, these data are consistent with the necessity of Akt-2 activation by a physiological dose of insulin for glucose homeostasis, as demonstrated in Akt-2-null mice (4), and it is Akt-2 activation through phosphorylation on Ser1474 that appears to be impaired in the patients during hyperglycemia.

The molecular mechanism of diminished Akt activation during hyperglycemia is not clear. Glucose, its metabolite glucosamine, and oxidative byproducts are powerful modulators of signal transduction and gene expression (6, 26, 36). Our data indicate that alterations in upstream insulin signaling are not involved, because InsR phosphorylation and expression of InsR, IRS-1/2, and PI3K were unchanged (Fig. 3), consistent with previous reports (30, 37, 40). Instead, our findings demonstrate that Akt-2 phosphorylation on Ser1474 was impaired during the hyperglycemic episode (Fig. 2). The impaired phosphorylation of Akt-2 on Ser1474 was associated with changes in the expression of specific proteins (Figs. 3 and 4; Table 2), including decreased expression of Akt-2 (Fig. 2) and possibly GLUT4 (Fig. 3). Interestingly, among the proteins in the patient muscle that correlated inversely with Akt-2 expression and Akt-2Ser1474 phosphorylation were several proteins with potential negative regulatory functions for insulin signaling (Table 2). Prominent among these were PKC-ε, PKC-θ, and the NF-κB-inhibitory kinase IκBα. Hyperglycemia and free fatty acids (FFAs) can increase synthesis of diacylglycerol, a cofactor for activation of conventional and novel PKCs (19). Treatment of skeletal muscle strips from obese, insulin-resistant patients with pharmacological inhibitors of conventional and novel PKCs causes significant augmentation of insulin-
mediated glucose uptake (8). FFA infusion can also induce insulin resistance, apparently through activation of inhibitory kinases of NF-κB (20). In mice lacking the IKKβ isoform, FFA infusion does not induce insulin resistance (23). Together, these data indicate that impaired glucose utilization by patients with atypical diabetes (44) is associated with a combination of factors that could contribute to insulin resistance: decreased Akt-2 expression, decreased Akt-2 phosphorylation, decreased GLUT4 expression, and expression of mechanisms counterregulatory to insulin action. The interdependencies among all of these factors will have to be determined by future studies.

In summary, we found that there was no association between glycemic status and Akt-1 expression or insulin-stimulated Akt-1 phosphorylation in muscle biopsies obtained from diabetic patients during hyperglycemic crisis and upon near-normoglycemic remission. However, a physiological concentration of insulin was unable to induce Akt-2 phosphorylation on Ser474 during the hyperglycemic period. In addition, the hyperglycemia was associated with decreased Akt-2 protein expression and increased expression of proteins with specific or potential counterregulatory roles for insulin signaling. Together, these data indicate that correction of hyperglycemia is associated with increased responsiveness of Akt-2 to physiological concentrations of insulin.

ACKNOWLEDGMENTS

We are grateful to L. A. Malinick for assistance with publication graphics. The assistance of National Eye Institute Vision Core (PHS 3P03 EY-13080) for antibody array scanning is greatly appreciated.

GRANTS

The research was supported, in part, by General Clinical Research Center Grant M01-RR-00211 and the American Diabetes Association (to G. E. Umpierrez).

REFERENCES