# Divergent Regulation of Akt1 and Akt2 Isoforms in Insulin Target Tissues of Obese Zucker Rats

**Young-Bum Kim, Odile D. Peroni, Thomas F. Franke, and Barbara B. Kahn**

**To determine whether impaired Akt (protein kinase B or rac) activation contributes to insulin resistance in vivo, we examined the expression, phosphorylation, and kinase activities of Akt1 and Akt2 isoforms in insulin target tissues of insulin-resistant obese Zucker rats. In lean rats, insulin (10 U/kg i.v. × 2.5 min) stimulated Akt1 activity 6.2-, 8.8-, and 4.4-fold and Akt2 activity 5.4-, 9.3-, and 1.8-fold in muscle, liver, and adipose tissue, respectively. In obese rats, insulin-stimulated Akt1 activity decreased 30% in muscle and 21% in adipose tissue but increased 37% in liver compared with lean littermates. Insulin-stimulated Akt2 activity decreased 29% in muscle and 37% in liver but increased 24% in adipose tissue. Akt2 protein levels were reduced 56% in muscle and 35% in liver of obese rats, but Akt1 expression was unaltered. Phosphoinositide 3-kinase (PI3K) activity associated with insulin receptor substrate (IRS)-1 or phosphotyrosine was reduced 67–86% in tissues of obese rats because of lower IRS-1 protein levels and reduced insulin receptor and IRS-1 phosphorylation. In adipose tissue of obese rats, in spite of an 86% reduction in insulin-stimulated PI3K activity, activation of Akt2 was increased. Maximal insulin-stimulated (100 nmol/l) glucose transport was reduced 70% in isolated adipocytes, with a rightward shift in the insulin dose response for transport and for Akt1 stimulation but normal sensitivity for Akt2. These findings suggest that PI3K-dependent effects on glucose transport in adipocytes are not mediated primarily by Akt2. Akt1 and Akt2 activations by insulin have a similar time course and are maximal by 2.5 min in adipocytes of both lean and obese rats. We conclude that *1*) activation of Akt1 and Akt2 in vivo is much less impaired than activation of PI3K in this insulin-resistant state, and *2*) the mechanisms for divergent alterations in insulin action on Akt1 and Akt2 activities in tissues of insulin-resistant obese rats involve tissue- and isoform-specific changes in both expression and activation. *Diabetes* 49:847–856, 2000**

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BSA, bovine serum albumin; $ED_{50}$, half-maximal dose; HA, hemagglutinin; IRS, insulin receptor substrate; NP-40, Nonidet p-40; PDK-1, PI(3,4,5)P3-dependent protein kinase 1; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase; PMSF, phenylmethylsulfonyl fluoride; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

The effect of insulin to acutely stimulate glucose uptake and metabolism in peripheral tissues is essential for normal glucose homeostasis. Resistance to this effect is a major pathogenic feature of type 2 diabetes (1,2) and contributes to the morbidity of obesity as well as type 1 diabetes (3). Whereas many of the proximal steps in insulin signaling have been identified, the downstream pathways for insulin action to maintain glucose homeostasis are still unknown. Insulin action involves a series of signaling cascades initiated by insulin binding to its receptor and eliciting receptor autophosphorylation and activation of receptor tyrosine kinases, which result in tyrosine phosphorylation of insulin receptor substrates (IRSs) (4–8). Phosphorylated IRSs activate phosphoinositide 3-kinase (PI3K), a necessary step for the stimulation of glucose transport by insulin (9–12). The latter is brought about primarily by translocation of the major insulin-responsive glucose transporter, GLUT4, from intracellular vesicles to the plasma membrane (4,13). The downstream pathways by which insulin-stimulated PI3K activation results in GLUT4 translocation remain unclear, but Akt serine threonine kinases are candidate molecules that could mediate the process. PI3K activation is necessary and sufficient for activation of Akt (protein kinase B or rac) by insulin and other growth factors (14–16). Furthermore, phosphorylation of Akt by lipid products of PI3K, such as phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3]-dependent protein kinase 1 (PDK-1), is an important step in Akt activation (17,18).

The importance of Akt activation for the metabolic actions of insulin is unclear. Data supporting a role for Akt include the fact that insulin administration in vivo in rats and humans rapidly activates Akt in skeletal muscle (19,20). The ability of Akt to inhibit glycogen synthase kinase-3 (19,21), a critical step in the activation of glycogen synthase by insulin, suggests a potential role for Akt in glycogen synthesis. In addition, Akt may regulate glycolysis via activation of phosphofructose 2-kinase (22). These effects deserve further study in vivo. The importance of Akt for insulin action on glucose transport remains unresolved. Overexpression of constitutively active Akt in adipocytes stimulates GLUT4 translocation to the plasma membrane (23,24), but overexpression of 2 dominant negative Akt mutants produces conflicting results (25,26).

The genes for 3 Akt isoforms have been cloned. Insulin has differential effects on these isoforms in a tissue- and species-specific manner. Insulin administration in rats rapidly activates Akt1 in skeletal muscle with minimal effect on Akt2 and no effect on Akt3 (27). In humans,

insulin activates all 3 isoforms in muscle, although the effect on Akt3 is small (20). Incubation of rat adipocytes in vitro with insulin results in activation of Akt1 and Akt2 but not Akt3, whereas in rat hepatocytes, in vitro insulin activates primarily Akt1 with very small effects on Akt2 and no effect on Akt3 (27). In human adipocytes, insulin activates Akt2 (28); effects on other isoforms have not been individually studied.

Data suggest that impairments in the initial steps of insulin signaling may play an important role in the insulin resistance associated with obesity and type 2 diabetes (29–37), but the roles of more distal steps such as Akt activation are unknown. Akt activation is impaired in adipocytes from humans with type 2 diabetes exposed to insulin in vitro (28). In vitro incubation of skeletal muscle from lean humans with type 2 diabetes shows a reduction of Akt activity at pharmacological but not physiological insulin concentrations (38). Insulin infusion in obese humans with type 2 diabetes normally activates all Akt isoforms in muscle in spite of 50% reduced activation of PI3K (20). In rats, however, hyperglycemia may interfere with insulin activation of Akt in muscle (39,40). The regulation of Akt in states of hyperinsulinemia without hyperglycemia has not been investigated. In addition, limited information is available regarding *1*) the role of individual Akt isoforms in insulin resistance, *2*) the mechanisms for changes in Akt isoform activity (e.g., alterations in gene expression vs. allosteric activation), and *3*) potential alterations in Akt activation in insulin target tissues other than muscle (e.g., fat and liver). The present study investigates the regulation of Akt1 and Akt2 expression and activation in insulin target tissues of obese Zucker rats.

## RESEARCH DESIGN AND METHODS

**Animal care and tissue harvest.** All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Female lean and obese *fa*/*fa* Zucker rats, 5 weeks old, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The rats were fed standard food (PMI Feeds, St. Louis, MO) and water ad libitum for 3 weeks. They were housed under controlled temperature at 24°C and a 12-h light-dark cycle with light from 0630 to 1830. For injection experiments, rats (8 weeks old) were fasted overnight. On the day of the experiment, they were anesthetized by inhalation of methoxyflurane (Pitman-More, Mundelein, IL), a bolus injection of insulin (10 U/kg) was administered through the jugular vein, and 2.5 min later gastrocnemius, liver, and periovarian fat tissue were rapidly removed, frozen in liquid nitrogen, and stored at –80°C until analysis. For experiments in isolated adipocytes, rats were not fasted.

**Determination of blood glucose and plasma insulin concentrations.** Blood samples were collected from the tail vein of rats in the fed state as previously described (41). Blood glucose levels were measured with a One Touch II glucose meter (LifeScan, Milpitas, CA). Plasma insulin concentrations were determined with a radioimmunoassay kit (Linco Research, St. Louis, MO).

**Preparation of Akt1 and Akt2 isoform-specific antibodies.** Antibodies specific for Akt1 or Akt2 were raised by immunization of rabbits with peptide sequences of mouse Akt1 (CKRQEEETMDFRSG) and Akt 2 (CYD SLGLLELDQRT). Neither of these sequences has significant homology to Akt3. The specificity of the antibodies was tested by transient transfection of COS-7 cells with the cDNAs for Akt1 or Akt2 fused to a hemagglutinin (HA) epitope tag (MYPYDVPDYASR) (16). COS-7 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 50 U/ml penicillin, and 50 µg streptomycin at 37°C, 5% $CO_2$. Cells were transfected at 80% confluence in serum-free medium using 2.5 µg HA-Akt1 or HA-Akt2 cDNA and 12.5 µl lipofectamine reagent (Life Technology, Rockville, MD) per 35-mm dish. Cells were harvested and solubilized 48 h after transfection. Proteins in lysates were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes. Akt1 and Akt2 were visualized by immunoblotting with antibodies specific for each isoform. The level of expression of the isoforms was normalized by immunoblotting with a monoclonal HA antibody (Boehringer Mannheim, Indianapolis, IN).

**Preparation of rat tissue lysates.** Tissues (50 mg) were homogenized using a polytron at half-maximum speed for 1 min on ice in 500 µl buffer A (20 mmol/l Tris, pH 7.5, 5 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 100 mmol/l NaF, 2 mmol/l $Na_3VO_4$) containing 1% Nonidet p-40 (NP-40), 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml aprotinin. Tissue lysates were solubilized by continuous stirring for 1 h at 4°C and centrifuged for 10 min at 14,000*g*. The supernatants were stored at –80°C until analysis.

**Determination of Akt activity.** Tissue lysates (500 µg protein) were subjected to immunoprecipitation for 4 h at 4°C with 5 µl Akt1- or Akt2-specific antibody (1:200 dilution) coupled to protein G-sepharose beads (Pharmarcia Biotechnology, Piscataway, NJ). Immune pellets were washed, and Akt activity was determined as previously described (20,42).

In time course studies, adipocytes were isolated as described below and preincubated for 30 min as a 10% suspension by volume in Krebs-Ringer HEPES buffer (20 mmol/l, pH 7.4) containing 2.5% bovine serum albumin (BSA) and 200 nmol/l adenosine and stimulated with 100 nmol/l insulin for 0, 2.5, 6.0, and 15 min. For dose-response experiments, adipocytes were incubated in the same buffer without (basal) or with (insulin-stimulated) 0.01–1,000 nmol/l crystalline porcine insulin. The cells were centrifuged through dinonylphthalate oil and resuspended in 500 µl buffer A containing 1% NP-40, 1 mmol/l PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Cell lysates were solubilized by continuous stirring for 1 h at 4°C and centrifuged for 10 min at 14,000*g*, and the supernatants were collected. Cleared lysates (70 µg protein) were used for Akt activity assays as described above.

**Determination of PI3K activity.** Tissue lysates (500 µg protein) were subjected to immunoprecipitation with 5 µl IRS-1 polyclonal antibody (1:100 dilution; gift from Dr. Morris White, Joslin Diabetes Center, Boston, MA) or monoclonal anti-phosphotyrosine antibody (4G10) (1:100 dilution; gift from Dr. Ronald Kahn, Joslin Diabetes Center) coupled to protein A-sepharose (Sigma, St. Louis, MO). The immune complex was washed, and PI3K activity was determined as described (20,42).

**Tyrosine phosphorylation of the insulin receptor and IRS-1.** Muscle lysates (1 mg protein) were subjected to immunoprecipitation overnight at 4°C with 5 µl anti-phosphotyrosine monoclonal antibody (4G10) coupled to protein A-sepharose. The immunoprecipitates were washed, and phosphorylation was visualized as described (42) using anti-phosphotyrosine monoclonal antibody at 1:200 dilution (Py 20; Santa Cruz Biotechnology, Santa Cruz, CA).

**Determination of Akt mobility shift and phosphorylation and p85 and IRS-1 protein content.** Tissue lysate protein (85–150 µg/lane) was resolved by SDS-PAGE (8% gel) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and incubated with the following antibodies: Akt1- or Akt2-specific polyclonal antibody (our antibodies as described above or Akt1 antibody from Santa Cruz Biotechnology and Akt2 antibody from Upstate Biotechnology [Lake Placid, NY]), a polyclonal antibody specific for Akt phosphorylated on ser 473, IRS-1 polyclonal antibody (gift from Dr. Ronald Kahn), or a polyclonal antibody against the p85α subunit of PI3K (Upstate Biotechnology) in 1% nonfat dry milk overnight at 4°C. The membranes were washed, and bands were visualized as described above and quantified by densitometry (Molecular Dynamics, Sunnyvale, CA).

**Glucose transport in isolated adipocytes.** Adipocytes were isolated from periovarian fat pads by collagenase digestion (1 mg/ml) (43,44). For dose-response studies, cells were incubated at 37°C with constant shaking in a 2% suspension by volume in Krebs-Ringer HEPES buffer (20 mmol/l, pH 7.4) with 2.5% BSA and 200 nmol/l adenosine and without (basal) or with (insulin-stimulated) 0.005–100 nmol/l crystalline porcine insulin. After the initial 30-min incubation with or without insulin, [U-$^{14}$C]glucose was added at a final concentration of 3 µmol/l for 30 min, and the reaction was terminated by separating cells from medium by spinning the suspension through dinonylphthalate oil (45). For time course studies, adipocytes were preincubated for 30 min in the same buffer, and 100 nmol/l insulin and 3 µmol/l [U-$^{14}$C]glucose were added for 2.5, 6.0, 15, or 30 min. Basal glucose transport rates were measured at all time points and showed no difference over time. Therefore, mean basal rates for each set of cells were determined, and nonspecific counts were subtracted to determine the transport rate at time 0 with insulin.

**Adipose cell size and number.** Isolated adipocytes were fixed with osmic acid and counted in a Coulter counter (Fullerton, CA) (46), and cell size (micrograms of lipid/cell) was calculated as previously described (46,47).

**Statistical analysis.** Data are presented as means ± SE. Statistical analyses including correlations were performed using the Statview program (Abacus Concepts, Berkeley, CA). Statistical significance was tested with repeated measures analysis of variance for activities and signaling assays (Figs. 1–5). For data in Table 1, Table 2, Fig. 3, and Fig. 5, unpaired Student's *t* test was used, since only 2 groups were compared.

TABLE 1
Characteristics of lean and obese Zucker rats

| | Lean | Obese |
|---|---|---|
| *n* | 6 | 7 |
| Body weight (g) | 166 ± 3 | 287 ± 11* |
| Periovarian fat pad (g) | 1.3 ± 0.3 | 12.6 ± 0.6* |
| Fat cell size (µg lipid/cell) | 0.18 ± 0.02 | 0.76 ± 0.08† |
| Gastrocnemius (g) | 1.90 ± 0.05 | 1.80 ± 0.05 |
| Glucose (mmol/l) | 4.35 ± 0.28 | 4.24 ± 0.06 |
| Insulin (pmol/l) | 347 ± 100 | 1,718 ± 289† |

Data are means ± SE. Blood glucose and plasma insulin concentrations were measured in the fed state. All rats were female. *$P < 0.01$ vs. lean rats; †$P < 0.001$.

## RESULTS

**Characteristics of lean and obese Zucker rats.** The body weight, periovarian fat pad weight, and fat cell size were greater in obese *fa*/*fa* rats at 8 weeks of age compared with their lean littermates (Table 1). Weight of the gastrocnemius muscle was not different between groups. Plasma insulin concentration of obese rats in the fed state was 5-fold higher than that of lean rats, but blood glucose was not different, indicating marked insulin resistance without frank diabetes.

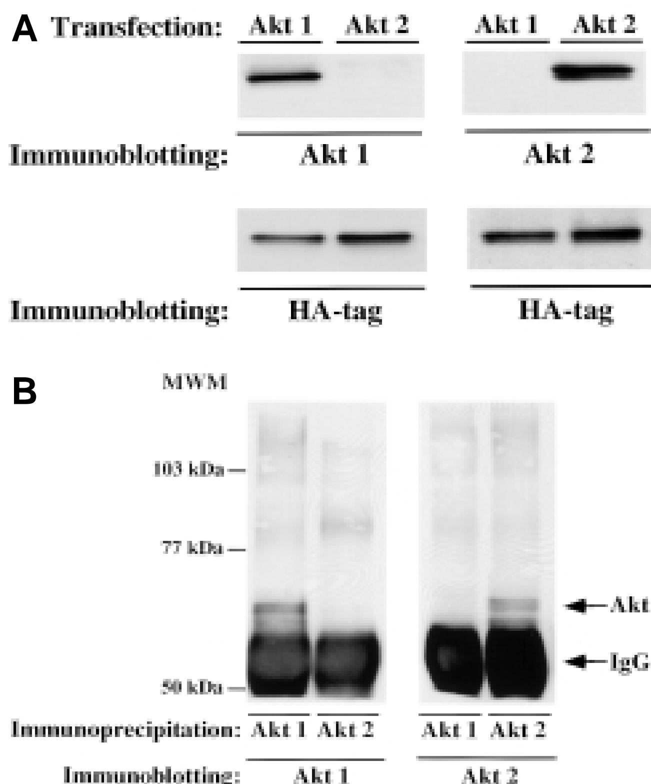


**FIG. 1.** ***A*: Specificity of Akt1 and Akt2 antibodies. The HA epitope tag was fused to the mouse Akt1 and Akt2 cDNAs (16), and each cDNA construct (2.5 µg) was transiently transfected into COS-7 cells using lipofectamine as described in RESEARCH DESIGN AND METHODS. Cells were solubilized 48 h later. Proteins in lysates were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes. Akt1 and Akt2 were visualized by immunoblotting with antibodies specific for each isoform and with an HA antibody. *B*: Specificity of immunoprecipitation with Akt1 and Akt2 antibodies. Proteins in skeletal muscle lysates (500 µg) were subjected to immunoprecipitation for 4 h at 4°C with antibodies specific for Akt1 or Akt2. The immunoprecipitated proteins were separated by SDS-PAGE on 8% gels and immunoblotted with the same Akt1 or Akt2 antibody. Molecular weight markers are indicated on the left. This figure is representative of 3 separate experiments.**

**Specificity of Akt1 and Akt2 antibodies.** Figure 1*A* shows the specificity of the antibodies raised against Akt1 and Akt2 isoforms. Expression plasmids containing HA-tagged Akt1 or Akt2 were transiently transfected in COS-7 cells, and proteins in lysates were separated by SDS-PAGE and immunoblotted using antibodies against Akt1, Akt2, or HA-tag. The upper left panel shows that the Akt1 antibody detected a band only in cells transfected with Akt1 and not in cells transfected with Akt2. The upper right panel shows that the Akt2 antibody detected a band only in cells transfected with Akt2 and not with Akt1. When the same lysates were blotted with an HA antibody, bands of similar intensity were seen (lower panels). Thus, both the Akt1 and Akt2 antibodies are highly specific for the respective isoforms, and there is no detectable cross-reactivity between them.

We also determined the specificity of immunoprecipitation with Akt1 and Akt2 antibodies, since Akt forms oligomers that could potentially involve a mixture of isoforms. Figure 1*B* shows Western blots of immunoprecipitates using either the Akt1 or Akt2 antibody. We detected Akt1 only in immunoprecipitates of the Akt1 antibody and not those of the Akt2 antibody. Similarly, we detected Akt2 only in immunoprecipitates of the Akt2 antibody and not those of the Akt1 antibody. Thus, immunoprecipitation with Akt1 and Akt2 antibodies is highly specific, and there is no cross-binding between the isoforms. With this protocol, we demonstrated by sequential immunoprecipitation that the efficiency of immunoprecipitation of Akt1 is ~84% and that of Akt2 is ~91% (not shown).

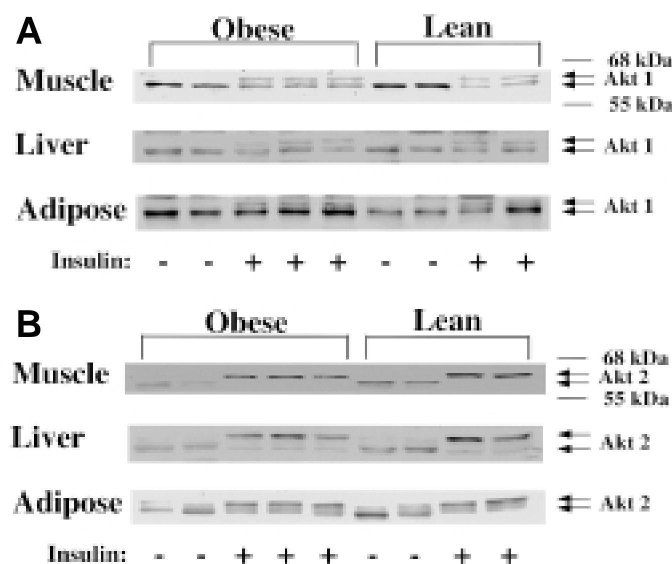


**FIG. 2. Akt1 (*A*) and Akt2 (*B*) mobility shift in muscle, liver, and adipose tissue from lean and obese Zucker rats. After an overnight fast, rats were injected intravenously with saline (–) or 10 U/kg insulin (+), and tissues were removed 2.5 min later. Proteins in tissue lysates were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes as described in RESEARCH DESIGN AND METHODS. Akt isoforms were visualized by immunoblotting with antibodies specific for either Akt1 or Akt2. Molecular weight markers are indicated on the right. Each lane contains tissues from a different rat. This autoradiogram is representative of 3 immunoblots for 6 lean and 7 obese rats.**

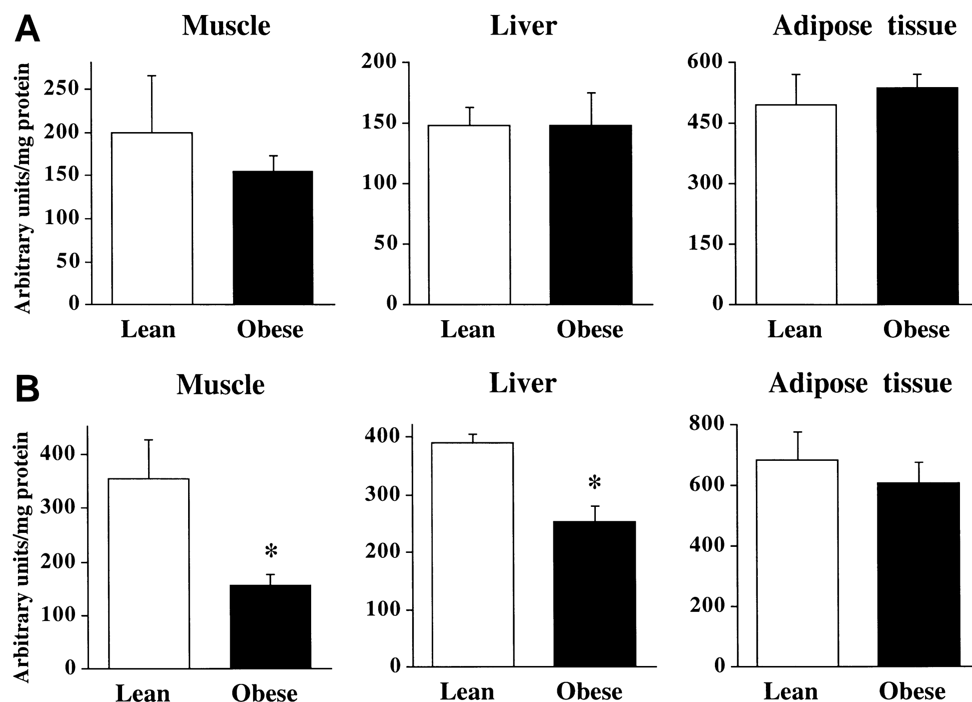


FIG. 3. Akt1 (*A*) and Akt2 (*B*) protein levels in muscle, liver, and adipose tissue from lean and obese Zucker rats. Proteins in tissue lysates were separated by SDS-PAGE on 8% gels and transferred to a nitrocellulose membrane. Akt isoforms were visualized by immunoblotting with antibodies specific for Akt1 or Akt2. Akt protein levels were quantitated using a densitometer. Data are means ± SE for 6–7 rats per group. $*P < 0.05$ vs. lean rats.

**Akt1 and Akt2 phosphorylation (mobility shift) and protein levels.** Three mammalian isoforms of Akt have been cloned: Akt1, Akt2, and Akt3. Unlike Akt1 and Akt2, the level of expression and activity of Akt3 is very low in insulin-sensitive tissues, and Akt3 is not activated by insulin in primary rodent tissues (27,48). Therefore, we focused on the Akt1 and Akt2 isoforms. Figure 2*A* shows the levels and insulin-stimulated hyperphosphorylation of Akt1 in tissues of obese and lean rats; Fig. 2*B* shows Akt2. In the basal state, we detected a single ~56-kDa band for Akt1 in muscle, liver, and adipose tissue. Akt2 appeared as a single band in muscle and liver and as a doublet with a faint upper band in adipose tissue even in the basal state in both obese and lean rats. After insulin stimulation of rats for 2.5 min, Akt1 and Akt2 shifted to a hyperphosphorylated state in both obese and lean rats. In muscle of obese and lean rats, only a portion of Akt1 became hyperphosphorylated in response to insulin, whereas all apparent Akt2 became hyperphosphorylated, with loss of the lower-molecular-weight species of Akt2. When we quantitated the bands by densitometry and expressed the results as the percent of total immunoreactive Akt in the hyperphosphorylated state, there were marked differences between isoforms and among tissues. In lean rats after insulin stimulation, in muscle 45 ± 2.9% of Akt1 was hyperphosphorylated, in liver 27.3 ± 4.4%, and in adipose tissue 22.5 ± 2.5%. After insulin stimulation of lean rats, 100% of Akt2 was hyperphosphorylated in muscle, 86 ± 1.4% in liver, and 61 ± 1.2% in adipose tissue. In obese rats, there was a significant reduction in the percent of Akt1 in muscle that became hyperphosphorylated in response to insulin (lean 45 ± 2.9%, obese 28 ± 1.6%; $P <$ 0.003; $n = 6$ lean and 7 obese).

Figure 3 shows the relative amounts of total (both phosphorylation states) of Akt1 and Akt2 proteins in each of the insulin target tissues of lean and obese rats. Whereas there was no significant change in the amount of Akt1 protein in any tissue of obese rats compared with lean (Fig. 3*A*), the amount of Akt2 decreased 56% in muscle and 35% in liver of obese rats (Fig. 3*B*).

**Activity of Akt1 and Akt2 isoforms in insulin target tissues.** Figure 4*A* shows that in lean rats, in vivo administration of insulin stimulated Akt1 activity 6.2-fold and Akt2 activity 5.4-fold in muscle, as measured by immune complex assay. In obese rats, there were no significant differences in basal activity of Akt1 or Akt2. However, the insulin-stimulated activity of both isoforms was reduced ~30% compared with lean littermates ($P < 0.01$), resulting in a 4-fold stimulation of Akt1 and 3-fold stimulation of Akt2. Figure 4*B* shows that in livers of lean rats, insulin stimulated Akt1 8.8-fold and Akt2 9.3-fold. In liver of obese rats, basal Akt1 activity tended to be increased, although it did not reach statistical significance. In obese rats, however, insulin-stimulated Akt1 activity in liver increased 37% ($P < 0.02$), whereas insulin-stimulated Akt2 activity was reduced 27% ($P < 0.05$). Although the changes in Akt1 activity were not associated with alterations in Akt1 protein levels in muscle or liver (Fig. 3), the decrease in Akt2 activity in muscle and liver of obese rats could be explained by the reduced Akt2 protein levels in these tissues as shown in Fig. 3. Figure 4*C* shows that in adipose tissue of lean rats, in vivo insulin administration stimulated Akt1 activity 4.4-fold and Akt2 activity 1.8-fold. In adipose tissue of obese rats, insulin-stimulated Akt1 activity was reduced 21% ($P < 0.05$), whereas Akt2 activity increased 24% ($P < 0.02$) compared with lean littermates. Unlike the regulation of Akt2 in muscle and liver, in adipose tissue of obese rats, the changes in Akt isoform activity did not appear to be due to altered expression of Akt1 or Akt2.

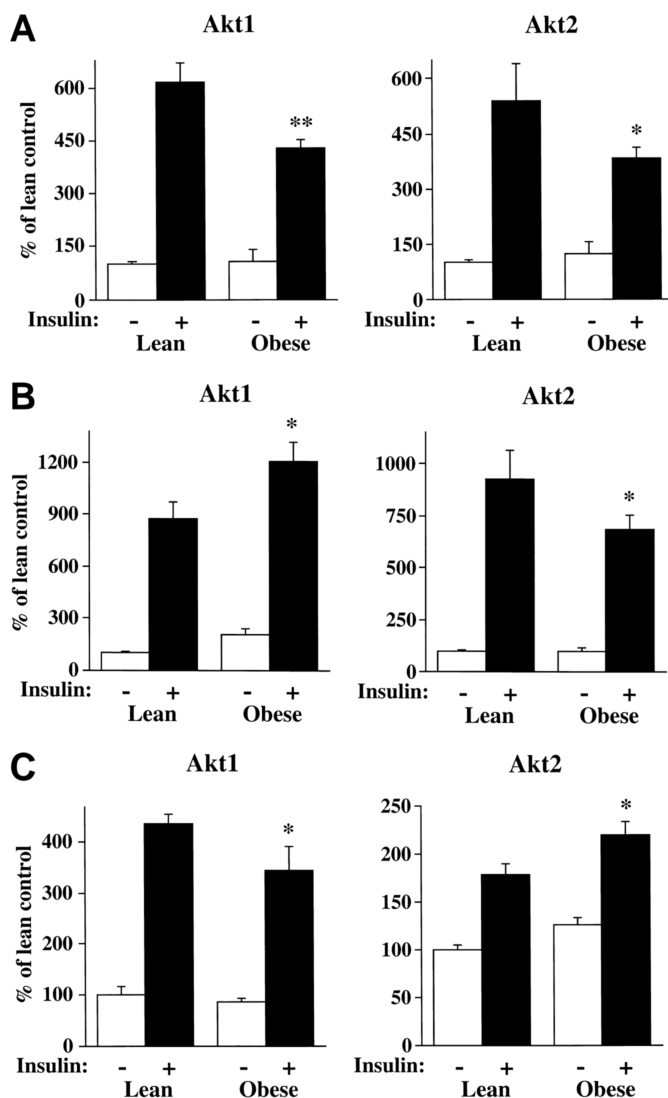


**FIG. 4. Akt1 and Akt2 activities in muscle (*A*), liver (*B*), and adipose tissue (*C*) from lean and obese Zucker rats. After an overnight fast, rats were injected intravenously with saline (–, □) or 10 U/kg insulin (+, ■), and tissues were removed 2.5 min later. Tissue lysates (500 µg) were subjected to immunoprecipitation with an antibody specific for Akt1 or Akt2. The immune pellets were assayed for kinase activity using crosstide as substrate. Data are means ± SE for 6 lean and 7 obese rats. *$P$ < 0.05 vs. lean insulin-injected rats; **$P$ < 0.01 vs. lean insulin-injected rats.**

We also measured phosphorylation of Akt with an antibody specific for phosphorylation on serine 473. This antibody has a higher affinity for phosphorylated Akt1 than for phosphorylated Akt2 (49). There was a striking 72% reduction of insulin-stimulated Akt phosphorylation in muscle, in agreement with the reduced gel shift we observed in Fig. 2. There was also a 42% decrease in insulin-stimulated phosphorylation of Akt in adipose tissue.

The relative abundance of Akt1 and Akt2 in a tissue cannot be definitively determined without extensive characterization of the affinity of each antibody for its respective isoform. However, we estimated the relative activity of the 2 Akt isoforms by comparing the counts per minute of Akt1 and Akt2 activity in the same amount of tissue protein. We first determined that both antibodies are relatively high affinity as judged by the high efficiency of immunoprecipitation: 84% for Akt1 and 91% for Akt2. Furthermore, transfection of the same amount of Akt1 and Akt2 cDNA in COS cells resulted in signals of similar intensity on Western blot when visualized with Akt isoform-specific antibodies and compared with bands visualized with an anti-HA epitope tag antibody (Fig. 1*A*). Thus, the Akt1 and Akt2 antibodies seem to have relatively similar affinities for the respective isoform. By comparing counts per minute of immunoprecipitated Akt1 or Akt2 activity, we determined that in lean rat muscle and liver, Akt1 activity was 37–81% higher than Akt2 activity, whereas in fat, Akt1 activity was 12- to 30-fold higher than Akt2 activity. Others have found Akt2 to be the predominant isoform in rat (50) and human (28) adipocytes. The differences may be due to different antibodies or protocols used.

**PI3K activity and p85 and IRS-1 protein levels.** Insulin stimulated IRS-1–associated PI3K activity 5.2- to 6.7-fold in muscle, liver, and adipose tissue in lean rats but only 1.7- to 2.5-fold in obese Zucker rats (Fig. 5*A*). Basal IRS-1–associated PI3K activity was unchanged, but insulin-stimulated activity was reduced 77% in muscle, 67% in liver, and 86% in adipose tissue of obese Zucker rats compared with lean littermates. PI3K activity was also measured in anti-phosphotyrosine immunoprecipitates from muscle lysates. In lean rats, insulin stimulated phosphotyrosine-associated PI3K activity 5.4-fold, whereas in obese rats, the stimulation was reduced to 2.8-fold ($P$ < 0.02, not shown). The amount of the p85 regulatory subunit of PI3K was unaltered in these insulin-target tissues of obese rats compared with lean littermates (Fig. 5*B*). In contrast, the total amount of IRS-1 protein was reduced 45% in muscle and 51% in adipose tissue ($P$ < 0.001) and tended to be reduced in liver of obese rats (Fig. 5*C*). The magnitude of the decrease in PI3K activity associated with IRS-1 was greater than the decrease in IRS-1 protein in all 3 tissues; this finding was especially pronounced in adipose tissue.

**Insulin receptor and IRS-1 tyrosine phosphorylation.** Because muscle is the major tissue responsible for insulin-stimulated glucose uptake in vivo, we first measured insulin receptor and IRS-1 tyrosine phosphorylation in skeletal muscle. Figure 6 shows that insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1 decreased 55% and 45%, respectively, in skeletal muscle of obese Zucker rats compared with lean littermates. The decrease in IRS-1 phosphorylation corresponds closely with the reduced amount of IRS-1 protein in obese rats. In parallel, the amount of p85 protein that was immunoprecipitated with IRS-1 antibody from muscle of insulin-stimulated rats decreased 48% in obese rats compared with lean littermates ($P$ < 0.05, not shown). Similar results were seen in other tissues.

**Dose-response of Akt1 and Akt2 isoform activity and glucose transport in adipose cells.** We investigated the dose-response of insulin-stimulated (6 min) Akt1 and Akt2 activity in isolated adipocytes from lean and obese Zucker rats. Insulin increased Akt1 activity in adipocytes from lean rats in a dose-dependent manner (Fig. 7*A*), with a maximal effect of 14.3-fold at 10 nmol/l insulin. In adipocytes from obese rats, insulin-stimulated Akt1 activity was reduced at all insulin concentrations ($P$ < 0.01 at 0.2 nmol/l; $P$ < 0.001 at 0.4–1,000 nmol/l), and maximally insulin-stimulated Akt1 activity decreased 70% compared with lean littermates. Akt2 activity increased in a dose-dependent manner (Fig. 7*B*), with a maximal stimulation of 1.4-fold at 1 nmol/l insulin in adipocytes from both lean

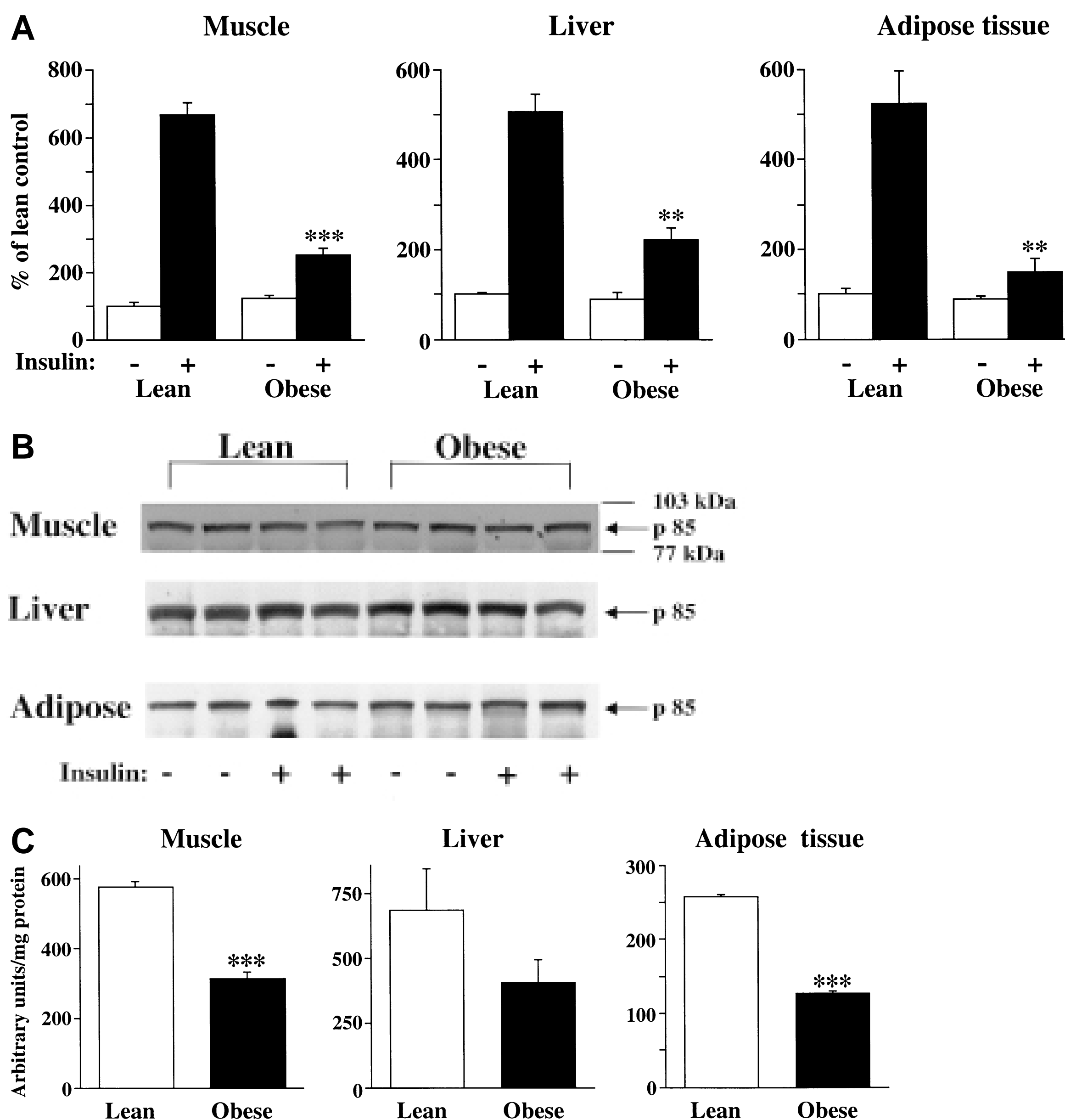


FIG. 5. *A*: PI3K activity in muscle, liver, and adipose tissue from lean and obese Zucker rats. After an overnight fast, rats were injected intravenously with saline (–, □) or 10 U/kg insulin (+, ■), and tissues were removed 2.5 min later. PI3K activity was measured in IRS-1 immunoprecipitates and quantitated using a PhosphorImager. Data are means ± SE for 6 lean and 7 obese rats. Similar data were obtained when PI3K activity was immunoprecipitated with an anti-phosphotyrosine antibody. $^{**}P < 0.01$ vs. lean insulin-injected rats; $^{***}P < 0.001$ vs. lean insulin-injected rats. *B*: p85 protein level in muscle, liver, and adipose tissue from lean and obese Zucker rats. Proteins in tissue lysates (100 μg/lane) were separated by SDS-PAGE on an 8% gel and transferred to nitrocellulose membrane. PI3K was visualized by immunoblotting with a p85α antibody. Molecular weight markers are indicated at the right of the upper blot. These autoradiograms are representative of 3 immunoblots for each tissue from a total of 6 lean and 7 obese rats. *C*: IRS-1 protein levels in muscle, liver, and adipose tissue from lean and obese Zucker rats. Proteins in tissue lysates were separated by SDS-PAGE on an 8% gel and transferred to nitrocellulose membrane. IRS-1 was visualized by immunoblotting with an IRS-1 antibody as described in RESEARCH DESIGN AND METHODS. Quantitation was performed with a densitometer. Data are means ± SE for 6–7 rats per group. $^{***}P < 0.001$ vs. lean rats; $P < 0.08$ for liver.

and obese rats. At very low and very high insulin concentrations, Akt2 activity tended to be increased in adipocytes from obese rats compared with lean littermates, but the difference was not as significant as in adipose tissue in vivo (Fig. 2*C*). In adipocytes from obese rats, the half-maximal dose ($ED_{50}$) for Akt1 stimulation increased compared with lean littermates, but that of Akt2 was unchanged (Table 2). In adipocytes from lean rats, Akt1 was less sensitive to insulin than Akt2 ($ED_{50}$ 77% higher, $P < 0.01$), but the magnitude of insulin stimulation of Akt1 was much greater than that of Akt2 (Fig. 7). Note the difference in *y*-axis scales in Fig. 7*A* and *B*.

To compare these signaling abnormalities directly with a metabolic endpoint, we measured insulin dose-response curves for glucose transport in the same adipose cell preparations from lean and obese Zucker rats (Fig. 7*C*). Insulin-stimulated glucose transport was decreased at all insulin concentrations ($P < 0.05$ at 0.4 nmol/l; $P < 0.001$ at 1–100 nmol/l) in adipocytes from obese rats. Maximally insulin-stimulated

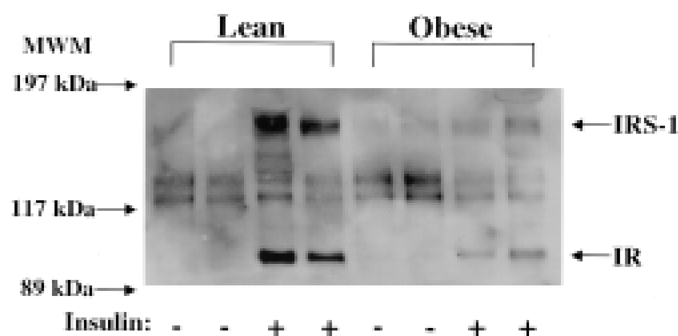


**FIG. 6. Tyrosine phosphorylation of the insulin receptor (IR) and IRS-1 in muscle of lean and obese Zucker rats. Tissues were removed 2.5 min after saline (–) or 10 U/kg insulin (+) intravenous bolus. Tissue lysates (1 mg protein) were subjected to immunoprecipitation with an anti-phosphotyrosine antibody (4G10). The precipitated proteins were separated by SDS-PAGE on a 6% gel and immunoblotted with an anti-phosphotyrosine antibody (Py20). Molecular weight markers are indicated on the left. This autoradiogram is representative of 3 immunoblots on a total of 6 lean and 7 obese rats.**

glucose transport was reduced 70% in obese rats compared with lean littermates. The $ED_{50}$ was increased in obese rats compared with lean littermates, indicating decreased insulin sensitivity (Table 2). There was a strong correlation between fat cell size and $ED_{50}$ for glucose transport in lean and obese rats ($y = 0.918x + 0.194$, $r^2 = 0.81$, $P < 0.006$). GLUT4 protein levels were reduced 63% in adipose tissue of obese rats (not shown), as previously reported (51). This decrease undoubtedly plays a major role in the decreased glucose transport in response to maximal insulin concentrations. However, the rightward shift in the dose response indicates an additional defect such as impaired signaling (52).

The $ED_{50}$ for glucose transport was similar to that for Akt2 stimulation in cells of lean rats (Fig. 7 and Table 2). In obese rats, the $ED_{50}$ for transport was shifted rightward (Fig. 7*C* and Table 2), whereas the $ED_{50}$ for Akt2 stimulation (Fig. 7*B*) was not. Together with the fact that maximal Akt2 stimulation was not impaired in cells from obese rats, these findings indicate that Akt2 activation is not sufficient for normal sensitivity of glucose transport stimulation by insulin.

**Time course of stimulation Akt1 and Akt2 isoform activity and glucose transport in adipose cells.** Basal Akt1 activity was similar in isolated adipocytes from lean and obese Zucker rats and remained the same from 0 to 15 min of incubation. The mean of the basal values for each group is shown as time 0 of insulin incubation. Insulin rapidly stimulated Akt1 activity 14-fold at 2.5 min, and thereafter the activation increased to 18.8-fold by 15 min in cells from lean rats (Fig. 8*A*). In adipocytes from obese rats, insulin-stimulated Akt1 activity increased only 6.4-fold at 2.5 min and did not increase further up to 15 min. The activation of Akt1 was impaired 58–70% at all time points in cells from obese rats compared with lean littermates ($P < 0.001$) (Fig. 8*A*). The magnitude of the impairment in Akt1 activity in adipocytes in vitro was greater than the decrease in adipose tissue in vivo (Fig. 4*C*). The greatest difference was actually in lean mice, in which Akt1 was stimulated ~4.3-fold in adipose tissue in vivo and 14- to 19-fold in isolated adipocytes. Most of the cells in fat pads are not adipose cells (53,54). Possibly, these abundant nonadipose cells, which are less insulin-sensitive, dilute the large stimulation of Akt1 in adipocytes and thereby diminish the difference between lean and obese when activity is measured in the intact fat pad.

In contrast to Akt1, insulin-stimulated Akt2 activity (Fig. 8*B*) in adipocytes increased only 1.6- to 2.0-fold at all time points in both lean and obese rats, and the stimulation was similar to that seen in intact fat tissues (Fig. 4*C*). Basal and insulin-stimulated Akt2 activity tended to be modestly increased at all time points in adipocytes from obese rats compared with lean littermates, but this difference did not reach statistical significance in isolated adipocytes as it did in adipose tissue (Fig. 4*C*).

For comparison, we investigated the time course of glucose transport in the same isolated fat cell preparations from lean and obese rats (Fig. 8*C*). In adipocytes from lean rats, insulin stimulated glucose transport to 64% of maximum at 6 min and to maximal levels by 15 min. In cells from obese rats, glucose transport was lower at all time points and reached maximal stimulation by 6 min. At 15 min, glucose transport in cells from obese rats was 68% lower than in lean rats. Thus, maximal stimulation of glucose transport occurs later than maximal

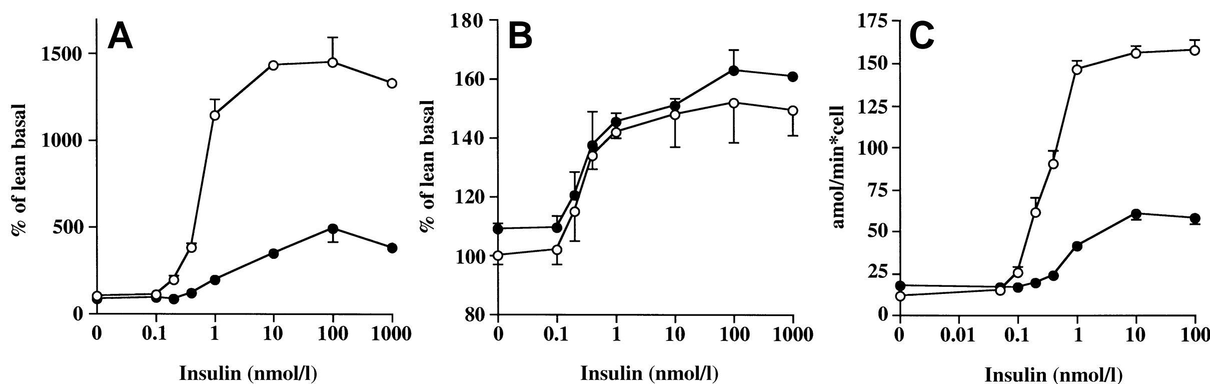


**FIG. 7. Dose response of Akt1 activity (*A*), Akt2 activity (*B*), and glucose transport (*C*) in isolated adipocytes from lean and obese Zucker rats. Periovarian fat pads were digested with collagenase. For Akt activity assays, cells were incubated in the absence or presence of insulin for 6 min. Cell lysates (70 µg) were subjected to immunoprecipitation with an antibody specific for Akt1 or Akt2. For measurement of [U-$^{14}$C]glucose uptake, cells were incubated in the absence or presence of insulin for 30 min. Each uptake assay was performed on cells from a single rat. Data are means ± SE for 3–4 rats per group. All Akt1 values in cells from obese rats incubated at ≥0.2 nmol/l insulin are different from lean rats at $P < 0.01$ or $P < 0.001$. All glucose transport values in cells from obese rats incubated at ≥0.4 nmol/l insulin are different from lean rats at $P < 0.05$ or $P < 0.001$.**

TABLE 2
Insulin concentrations for $ED_{50}$ values of Akt isoform activity or glucose transport in isolated adipocytes

| | Lean | Obese | *P* (lean vs. obese) |
|---|---|---|---|
| Akt1 activity | 0.69 ± 0.03 | 1.77 ± 0.18* | <0.001 |
| Akt2 activity | 0.39 ± 0.06† | 0.47 ± 0.11‡ | NS |
| Glucose transport | 0.35 ± 0.19§ | 0.85 ± 0.14*‖¶ | <0.01 |

Data are means ± SE. *Significant difference for same measurement compared with lean control rats; †$P < 0.01$ between Akt1 and Akt2 activity in lean control rats; ‡$P < 0.001$ between Akt1 and Akt2 activity in obese rats; §$P < 0.01$ between Akt1 activity and glucose transport in lean control rats; ‖$P < 0.001$ between Akt1 activity and glucose transport in obese rats; ¶$P < 0.05$ between Akt2 activity and glucose transport in obese rats.

stimulation of Akt1 or Akt2, as would be expected if transport were downstream of Akt.

**DISCUSSION**

We investigated the possibility that impaired activation of Akt1 or Akt2 contributes to insulin resistance in obese Zucker *fa/fa* rats. We demonstrated isoform- and tissue-specific regulation of Akt1 and Akt2 in insulin target tissues of these rats. We also found a discrepancy between the impairment in insulin activation of PI3K and of Akt isoforms. In vivo in all tissues, insulin action on Akt isoforms is better preserved than insulin action on PI3K, and in some tissues, activity of Akt1 or Akt2 is even increased. Similar discrepancies in PI3K and Akt activities have recently been reported with insulin infusion in obese humans with type 2 diabetes (20) and in several insulin-resistant models in rats (39,40,42). These findings suggest the presence of either PI3K-independent pathways that regulate Akt activity in insulin-resistant states or regulatory steps distal to the activation of PI3K. PI3K-independent activation of Akt has been reported in response to growth hormone (55), isoproterenol (56), osmotic shock (57), and changes in intracellular calcium (58); the mechanisms are unknown. Distal regulation of Akt could involve PDKs (17,18) or the phosphatases that act on the phospholipid products of PI3K such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a dual specificity phosphatase that dephosphorylates $PI(3,4,5)P_3$ (59). Upregulation of PDKs or downregulation of the expression or activity of molecules such as PTEN could result in relatively preserved or even increased Akt activation when PI3K activity is markedly decreased. Recent data indicate that a *C. elegans* PTEN homolog acts in an insulin receptor–like metabolic signaling pathway, suggesting that mammalian PTEN might modulate insulin signaling (60). Although another possible explanation of our data is that very little PI3K activity is required for full activation of Akt, that would not explain the opposite changes in PI3K and Akt activities in some tissues of obese Zucker rats.

Some changes in Akt activity in tissues of obese rats can be explained by altered expression of Akt isoforms, whereas others cannot and are most likely due to altered allosteric activation. The differences in the regulation of Akt isoforms could be explained by the involvement of different PDKs or by distinct intracellular localization of these Akt isoforms. In fact, Akt1 and Akt2 have been reported to have different subcellular distributions in adipocytes (61,62). Differential regulation is also evident from the fact that expression of Akt2 but not Akt1 is induced with differentiation of muscle cells (63) and adipocytes (64). The different degrees of phosphorylation of Akt1 and Akt2 in different tissues (Figs. 2 and 3) also suggest different regulatory factors for these isoforms.

Akt2, but not Akt1, was recently found to be recruited to (61,62) and phosphorylated in (62) GLUT4-containing vesicles in adipocytes stimulated with insulin. Therefore, Akt2 has been hypothesized to play a role in insulin-stimulated GLUT4 translocation in adipocytes. However, we found normal or slightly increased Akt2 activity in adipocytes of insulin-resistant obese rats, even though both the maximal response and the sensitivity of insulin-stimulated glucose transport were reduced. Although the reduction in maximal stimulation of transport is largely due to decreased GLUT4 expression, the defect in insulin sensitivity most likely reflects a signaling defect (52). If Akt2 were the main downstream signal, the $ED_{50}$ for transport should be normal in obese rats, since Akt2 stimulation is normal. In contrast, Akt1 sensitivity to insulin and maximal stimulation (Fig. 7*A*) are decreased in obese rats, making Akt1 more likely than Akt2 to be involved in the glucose transport

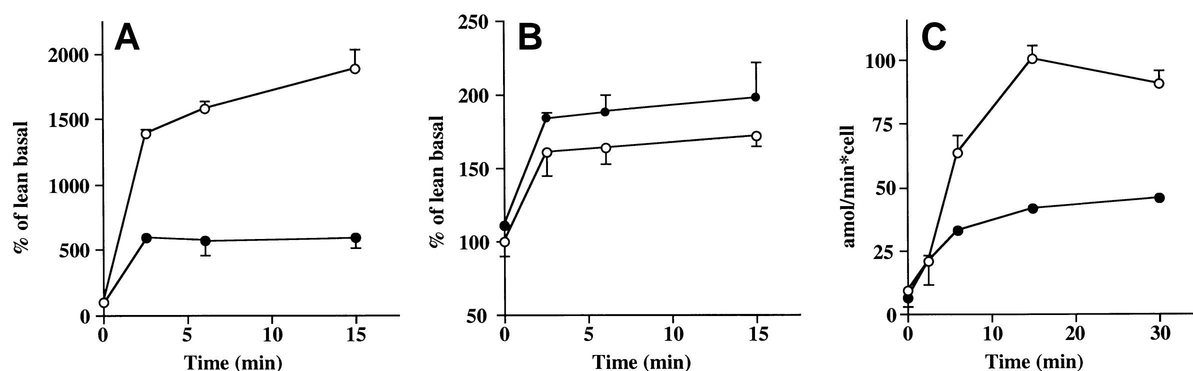


**FIG. 8. Time course of Akt1 activity (*A*), Akt2 activity (*B*), and glucose transport (*C*) in isolated adipocytes from lean and obese Zucker rats. Periovarian fat pads were digested with collagenase and Akt activity or [U-$^{14}$C]glucose transport was measured at 2.5, 6.0, 15.0, and 30 min in the presence of 100 nmol/l insulin as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 3–4 rats per group. Akt1 activity at all time points is different in lean and obese rats at $P < 0.001$. Glucose transport is different at 6.0, 15.0, and 30 min in lean and obese rats at $P < 0.001$.**

defect. However, at the $ED_{50}$ (0.35 nmol/l) for glucose transport in adipocytes from lean rats, stimulation of Akt1 is minimal, indicating either that very low levels of Akt1 are required to stimulate glucose transport or that Akt1 is not involved. Thus, these data do not support a critical role for either Akt isoform in insulin action on glucose transport in rat adipocytes.

Unlike in adipocytes, in skeletal muscle of obese rats, insulin-stimulated activities of Akt1 and Akt2 are reduced ~30%. This reduction is unlikely to impair insulin-stimulated glucose transport, since recent studies in models of insulin resistance induced with hyperglycemia in vivo (39) or in vitro (40) show that an ~40% reduction in insulin-stimulated Akt activation in skeletal muscle is associated with no defect in insulin-stimulated glucose transport. On the other hand, glucose metabolism to glycogen is impaired (40), potentially indicating differential dependency of glucose transport and glycogen synthesis on Akt activation. The reduction of Akt activity in muscle of obese rats contrasts with the normal activity in muscle of obese humans with and without type 2 diabetes (20). The difference may be due to the mild degree of insulin resistance in the obese nondiabetic humans studied (20) or differences in the dose and duration of insulin treatment in these studies. Pharmacologically high insulin concentrations appear to bring out defects that are not present at high physiological concentrations (38). Regardless, the conclusion is similar—defects in the activity of Akt isoforms are unlikely to play a major role in resistance to insulin-stimulated glucose transport in muscle in obese and diabetic states.

In summary, this is the first demonstration of the regulation of specific Akt isoforms in an altered metabolic state. Surprisingly, whereas activation of PI3K is markedly impaired in all insulin target tissues, Akt expression, phosphorylation, and activity undergo tissue- and isoform-specific regulation. Some changes in insulin-stimulated activity of Akt isoforms can be explained by altered expression, but others are most likely due to altered allosteric activation. In all insulin target tissues of obese rats, the changes in Akt1 or Akt2 activity in vivo are smaller than the impairment in PI3K activity, and in some tissues, the activity of an Akt isoform is increased. These findings suggest that either a PI3K-independent pathway is involved in stimulation of Akt by insulin in vivo or other factors can modulate the effect of PI3K on Akt.

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