Functional Studies of Akt Isoform Specificity in Skeletal Muscle in Vivo; Maintained Insulin Sensitivity Despite Reduced Insulin Receptor Substrate-1 Expression

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The phosphoinositide 3-kinase/Akt pathway is thought to be essential for normal insulin action and glucose metabolism in skeletal muscle and has been shown to be dysregulated in insulin resistance. However, the specific roles of and signaling pathways triggered by Akt isoforms have not been fully assessed in muscle in vivo. We overexpressed constitutively active (ca-) Akt-1 or Akt-2 constructs in muscle using in vivo electrophoresis and, after 1 wk, assessed the roles of each isoform on glucose metabolism and fiber growth. We achieved greater than 2.5-fold increases in total Ser473 phosphorylation in muscles expressing ca-Akt-1 and ca-Akt-2, respectively. Both isoforms caused hypertrophy of muscle fibers, consistent with increases in p70S6kinase phosphorylation, and a 60% increase in glycogen accumulation, although only Akt-1 increased glycogen synthase kinase-3β phosphorylation. Akt-2, but not Akt-1, increased basal glucose uptake (by 33%, P = 0.004) and incorporation into glycogen and lipids, suggesting a specific effect on glucose transport. Consistent with this, short hairpin RNA-mediated silencing of Akt-2 caused reductions in glycogen storage and glucose uptake. Consistent with Akt-mediated insulin receptor substrate 1 (IRS-1) degradation, we observed approximately 30% reductions in IRS-1 protein in muscle overexpressing ca-Akt-1 or ca-Akt-2. Despite this, we observed no decrease in insulin-stimulated glucose uptake. Furthermore, a 68% reduction in IRS-1 levels induced using short hairpin RNAs targeting IRS-1 also did not affect glucose disposal after a glucose load. These data indicate distinct roles for Akt-1 and Akt-2 in muscle glucose metabolism and that moderate reductions in IRS-1 expression do not result in the development of insulin resistance in skeletal muscle in vivo. (Molecular Endocrinology 21: 215–228, 2007)
dition, phosphorylation of muscle Akt is reduced in some models of lipid-induced insulin resistance (11, 12), but the effect seems to be predominantly on Akt-2 (13, 14).

There are several possible explanations for the isoform-specific effects of Akt. One possibility is that tissue-specific expression governs their unique biological roles. For example, it has been shown that whereas Akt-1 is the dominant isoform in 3T3-L1 preadipocytes, during differentiation the expression of Akt-1 declines in parallel with an increased expression of Akt-2 (15). Alternatively, different Akt isoforms may couple to unique downstream targets either via discrete subcellular localization or by binding to distinct scaffolding proteins (16). Thus, although there is some evidence from in vitro and whole organism knockout studies to suggest a basis for differential activities of Akt isoforms in muscle, separate roles for these isoforms have not been established under well-defined experimental conditions in muscle in vivo. In vivo electrottransfer (IVE) is a means for short-term genetic manipulation of single skeletal muscles and provides these conditions as it avoids the potential confounding effects of developmental adaptation to germ-line manipulation and whole-body changes in insulin sensitivity that are common with global knockout or transgenic approaches (17).

A negative feedback effect of chronic stimulation of Akt or its downstream effectors mammalian target of rapamycin (mTOR) and p70S6kinase upon IRS-1 has been shown to result in muscle insulin resistance (18–22). This negative feedback is thought to be the result of serine phosphorylation of IRS-1 resulting in inhibition of tyrosine phosphorylation and thus membrane recruitment of phosphoinositide-3 kinase plus increased targeting of IRS-1 to the proteasomal degradation pathway. However, despite the volume of correlative and knockout (23) evidence suggesting the importance of an impairment in IRS-1 per se in the development of insulin resistance, there is little direct evidence to support this contention in an in vivo setting.

In the present study we have separately overexpressed constitutively active versions of Akt-1 and Akt-2 isoforms in skeletal muscle of rats using IVE and investigated their specific effects on downstream signaling, glucose metabolism, and insulin action.

**RESULTS**

**Specific Local Overexpression of Akt-1 and -2 Isoforms in Skeletal Muscle**

EH114-Akt-1 and EH114-Akt-2 constructs were administered by electrottransfer into the right tibialis cranialis (TC) muscles of separate cohorts of rats concurrent with administration of EH114-green fluorescent protein (GFP) vector into the left TC muscles. After 1 wk constitutively active (ca)-Akt-1 and -2 overexpression was confirmed by immunoblotting of muscle lysates using an anti-Ser473 Akt antibody that recognizes the active phosphorylated form of the kinase. As can be seen in Fig. 1A, bands of approximate molecular mass 40 and 47 kDa corresponding to the predicted molecular masses of the constitutively active myristoylated Akt-1 and -2 proteins, respectively (24), were detected in the test muscle lanes only. In addition, immunoreactive bands of apparent molecular mass 55 kDa were detected in both test and control muscles, corresponding to the endogenous Akt isoforms. Total pS473-phosphorylation of Akt in each muscle was determined by addition of the density readings of the two specific bands, and this total was increased by more than 150% in both ca-Akt-1– (Fig. 1B) and ca-Akt-2–electroporated muscles vs. paired controls (Fig. 1C). The truncated ca-Akt-1 protein was also recognized by an anti-Akt-1-specific antibody and by a pan-Akt antibody, whereas we were unable to detect the ca-Akt-2 isoform using these antibodies due to disruption of the specific epitopes they recognize in this mutant. In Akt-1-overexpressing muscles these antibodies revealed a 308% increase in total Akt-1 protein and a 320% increase in total Akt protein vs. paired control muscles; Fig. 1A).

Transverse sections from ca-Akt-1, ca-Akt-2, and control muscles were immunostained for myosin heavy chain to determine whether Akt overexpression resulted in altered muscle fiber type distribu-
There were no differences observed in the percentage of type I fibers between test and control muscles (3.9% Akt-1 vs. 3.6% paired control; 3.0% Akt-2 vs. 3.1% paired control). These percentages are consistent with those previously quoted (25). Representative sections for Akt-1 rats are displayed in Fig. 2, F and G.

Akt-2 Selectively Regulates Glucose Disposal in Muscle

Prior studies have assessed the consequences of Akt overexpression on muscle fiber size (3, 5). However, these studies did not assess the role of Akt in muscle glucose metabolism, nor did they consider the differential effects of Akt isoforms. Thus, to characterize the effects of Akt overexpression on glucose disposal and storage in muscle, test and control Akt-1 and Akt-2 electroporated muscles were assayed for glycogen and triglyceride content and acute rates of glucose uptake and incorporation into glycogen and lipids under conditions of basal circulating insulin. Mean plasma whole-body glucose disposal (Rd) values for ca-Akt-1 and ca-Akt-2 electroporated rats were similar (7.7 ± 0.8 mg/kg-min and 6.8 ± 0.5 mg/kg-min, respectively). Muscles overexpressing ca-Akt-1 and ca-Akt-2 did not exhibit any significant difference in muscle triglycerides (data not shown) whereas glycogen levels were increased by 56 and 61% (P < 0.001), respectively. Overexpression of each Akt isoform had
disparate effects on acute muscle glucose disposal. Despite showing a high degree of expression, ca-Akt-1 overexpression had no significant effect on basal glucose uptake (Fig. 3A) or on the acute incorporation of glucose into glycogen (Fig. 3B) or lipids (Fig. 3C). In contrast, overexpression of ca-Akt-2 resulted in a 36% increase in glucose uptake (Fig. 3D; \(P = 0.021\)), a 59% increase in incorporation of glucose into glycogen (Fig. 3E; \(P < 0.001\)), and a 108% increase in incorporation into lipids (Fig. 3F; \(P = 0.016\)). These effects occurred in the absence of any differences in protein expression of the glucose transporter GLUT-4 in rats electroporated with either Akt isoform (data not shown). These findings emphasize the specific importance of the Akt-2 isoform for muscle glucose metabolism.
Akt Overexpression Induces Phosphorylation of Glycogen Synthase Kinase and p70S6 Kinase

In an attempt to provide a mechanistic basis for the effects of Akt overexpression on muscle fiber hypertrophy and glycogen storage, Western analysis of muscle lysates was undertaken to determine the phosphorylation status of key signaling molecules downstream of Akt. Phosphorylation of glycogen synthase kinase (GSK)-3β at serine 9 was increased by Akt-1 overexpression by 39% (Fig. 4A; \( P < 0.001 \)); however, there was no significant change in GSK-3β phosphorylation in muscles overexpressing Akt-2 (Fig. 4E). In the former case this could be an explanation for the observed increase in glycogen storage, because phosphorylation at this site is inhibitory and is responsible for removal of the inhibitory effect of GSK-3β on glycogen synthase (5, 26). Akt activation has also been shown to trigger activation of the mTOR-p70S6 kinase pathway, which leads to muscle fiber hypertrophy (3, 5). Although there was a trend toward an increase in pS2448-mTOR in ca-Akt-1 muscles (40%, \( P = 0.068 \); Fig. 4B), there was no such effect in ca-Akt-2 muscles (Fig. 4F), and indeed total mTOR expression was marginally decreased in ca-Akt-2 muscles (by 17%, \( P = 0.04 \); Fig. 4G). However, an effect of Akt overexpression on mTOR phosphorylation was also not reported in the study by Bodine et al. (3, 5). Nevertheless, p70S6 kinase phosphorylation at the mTOR site (Thr389) was markedly increased by 200% in ca-Akt-1 muscles (\( P < 0.001 \); Fig. 4D) and by 53% (\( P = 0.044 \); Fig. 4H) in muscles overexpressing ca-Akt-2. The con-
sequent differences in GSK (5) or p70S6 kinase activity generated as a result of Akt-1 and Akt-2 activation may be related to the more modest effect of the latter isoform upon fiber hypertrophy shown in Fig. 2.

Short Hairpin (sh)RNA-Mediated Akt-2 Gene Silencing in Rat Muscle Impairs Insulin-Stimulated Glucose Disposal and Glycogen Storage

In an attempt to confirm our finding that Akt-2 specifically mediates glucose disposal into skeletal muscle, we performed the complementary experiment, in which we administered vectors expressing three shRNAs targeting distinct Akt-2 mRNA sequences and investigated the effect of specific gene silencing on glycogen storage and insulin-stimulated glucose disposal into test and control muscles under hyperinsulinemic-euglycemic clamp conditions 1 wk later. Immunoblotting of muscle lysates confirmed that Akt-2 expression was decreased by 31% ($P < 0.004$) and total pS473-Akt by 35% ($P = 0.002$), whereas Akt-1 protein was not affected by the manipulation (Fig. 5, A–C). As a result of this, glycogen storage at the end of 1 wk was reduced by 31% ($P < 0.001$) (Fig. 5D), whereas total glucose disposal and glucose incorporation into glycogen assessed by tracer kinetics were inhibited by 35% ($P = 0.001$) and 51% ($P = 0.019$), respectively (Fig. 5, E–F). These data corroborate the findings obtained through ca-Akt-2 overexpression and are in agreement with previous knockout mouse data in which deletion of Akt-2, but not Akt-1, resulted in muscle insulin resistance (8, 9). Collectively, these data confirm the requirement of Akt-2 for insulin-stimulated glucose disposal into skeletal muscle.

Chronic Akt Activation Causes a Reduction in IRS-1 Phosphorylation and Expression

Chronic insulin stimulation has been shown to cause insulin resistance via a negative feedback loop involving serine phosphorylation of the adaptor protein...
IRS-1 by a kinase pathway including mTOR and p70S6 kinase. This leads to a net reduction in IRS-1 levels due to its proteasomal degradation (27–29). However, it is unclear which Akt isoform mediates this effect or indeed whether this effect is specific to the Akt pathway. To determine this we measured IRS-1 protein expression and phosphorylation in each set of muscles described above. Basal phosphorylation of IRS-1 at Tyrosine 612 was reduced by both ca-Akt-1 (Fig. 6A) and ca-Akt-2 (Fig. 6D) overexpression, by 33% (P < 0.01) and 24% (P = 0.032), respectively. Consistent with this, total IRS-1 protein expression was reduced by a similar amount in each paired set of muscles. IRS-1 expression was reduced by 33% by ca-Akt-1 overexpression (Fig. 6B; P = 0.015) and by 27% in ca-Akt-2 muscles (Fig. 6E; P = 0.008). Because phosphorylation at serine 307 in IRS-1 has been implicated in muscle insulin resistance (22, 27, 28), we also assessed the degree of phosphorylation at this site. However, as can be seen in Fig. 6C, we found no effect of chronic Akt activation on this parameter. Thus, despite showing an Akt-dependent effect on IRS-1 levels in muscles, we were unable to detect an isoform-specific effect on this parameter. Furthermore, there was no effect of either Akt-1 or Akt-2 overexpression on IRS-2 expression (mean band intensities Akt-1 0.76 vs. control, 0.75 arbitrary units; Akt-2 1.96 vs. control, 2.01 arbitrary units), suggesting that constitutive activation of Akt had no negative feedback effect upon this molecule and that increased

![Fig. 5. Akt-2 Knockdown in Muscle Causes Local Reductions in Glycogen Storage and Insulin-Stimulated Glucose Uptake](image-url)

Western blots and summary graphs showing mean reductions of 31% and 35% in Akt-2 protein (A) and total pS473-Akt (B) in muscles electroporated with two specific Akt-2 shRNAs, with no effect of the manipulation on Akt-1 protein (C). This resulted in a 31% decrease in stored glycogen (D) and 51% and 35% reductions in glucose tracer incorporation into glycogen (E) and glucose disposal (F), respectively, under hyperinsulinemic-euglycemic clamp conditions. **, P < 0.01; ***, P < 0.001 vs. control muscle. C, Control; T, test.
expression of IRS-2 was not induced to compensate for the reduction in IRS-1 protein.

**Chronic Akt Activation in Muscle Has No Effect on Local Insulin Sensitivity**

In view of the reduction in IRS-1 levels upon overexpression of ca-Akt, we wanted to assess the hypothesis recently put forward (18–22), that this negative feedback on the proximal insulin signaling cascade results in muscle insulin resistance. Therefore, separate cohorts of rats were electroporated with Akt-1 or Akt-2 expressing constructs as previously, and glucose disposal into each muscle was measured under euglycemic-hyperinsulinemic clamp conditions 1 wk later. This round of electrotransfer resulted in a mean

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**Fig. 6. Chronic Akt Activation Causes IRS-1 Down-Regulation in Muscle**

Western blots and summary graphs demonstrating that Akt-1 (A) and Akt-2 (D) overexpression resulted in 33% and 24% reductions in tyrosine phosphorylation of IRS-1, respectively. This was reflected in similar 33% and 27% reductions in protein expression of IRS-1 in Akt-1 (B)- and Akt-2 (E)-overexpressing muscle. However, no effect of either isoform was recorded in serine phosphorylation of IRS-1 at position 307 (C). *, $P < 0.05$; **, $P < 0.01$ vs. control muscle. O/E, Overexpression; C, control; T, test.
96% increase in Ser473-Akt phosphorylation in muscles electroporated with Akt-1 ($P = 0.038$) and a 77% increase in muscles electroporated with Akt-2 ($P < 0.001$). $R_d$ was $32.6 \pm 2.6$ mg/kg-min and $31.7 \pm 1.2$ mg/kg-min in ca-Akt-1 and ca-Akt-2 electroporated rats, respectively. Despite the chronic Akt activation and a reduction in IRS-1 of a magnitude equivalent to that observed after several weeks’ high-fat feeding (30), there were no differences detected in insulin-stimulated glucose uptake in ca-Akt-1 (Fig. 7A) or ca-Akt-2-overexpressing muscles (Fig. 7B).

shRNA-Mediated Silencing of IRS-1 in Mouse Muscle Does Not Impair Glucose Disposal

Having established that a moderate decrease in IRS-1 expression is insufficient to impair insulin-stimulated glucose disposal into muscle, we wished to determine whether a more profound IRS-1 silencing would show an altered end point. To this end we used IVE to simultaneously express two shRNAs targeting distinct IRS-1 mRNA sequences in one TC muscle and a shRNA targeting luciferase (nonsense) in the contralateral TC muscle. One week later we assessed IRS-1 protein and glucose tracer clearance into muscles as part of an ip glucose tolerance test (IPGTT). This manipulation reduced IRS-1 protein by a mean 68% ($P < 0.001$; Fig. 8A) and IRS-1 tyrosine phosphorylation by 53% ($P < 0.001$; Fig. 8B), an effect that is equal to or greater in magnitude than that seen in the Zucker fatty rat, a model of extreme obesity and insulin resistance (31). However, despite the magnitude of this effect, we were still unable to detect any difference in glucose clearance to paired muscles after administration of a glucose load (Fig. 8C), implying a lack of a causative effect of IRS-1 degradation on insulin sensitivity in muscle.

DISCUSSION

In the present study we have attempted to map the specific downstream effects and targets of Akt-1 vs. Akt-2 in skeletal muscle in vivo using an approach that avoids the confounding effects of developmental adaptation and changes in whole-body physiology inherent in traditional transgenic approaches (17). Such a contemporaneous assessment of the relative roles of each Akt isoform in muscle with particular regard to their roles in glucose metabolism has not been performed to date in vivo. It has previously been shown that engineering a membrane targeting motif into Akt results in its constitutive activation (24), and herein IVE has been employed to separately express constitutively active Akt-1 and Akt-2 constructs in a specific muscle. We have found that overexpression of both ca-Akt-1 and ca-Akt-2 results in significant muscle hypertrophy and increased glycogen mass. However, Akt-2 had a much more robust effect on muscle metabolism as estimated by glucose uptake or glucose incorporation into lipid and/or glycogen in models of both Akt-2 overexpression and silencing. Intriguingly, only Akt-1 overexpression resulted in a significant increase in phosphorylation of GSK and had a more marked effect on p70S6kinase. In contrast, Akt-2 appeared to have a more predominant role in muscle glucose disposal, and its overexpression demonstrated an acute effect on glycogen synthesis without any detectable change in GSK-3 phosphorylation.

Previous studies have shown that overexpression of constitutively active Akt-1 in muscle causes hypertrophy (3). Our data are consistent with these observations and further show that this effect is not restricted to the Akt-1 isoform but also occurs after Akt-2 overexpression. Intriguingly, at 7 d after electrotransfer, we observed a robust increase in p70S6kinase phosphor-

Fig. 7. Chronic Akt Activation Has No Effect on Insulin-Stimulated Glucose Uptake into Muscle

Graphs demonstrating that under hyperinsulinenic-euglycemic clamp conditions there were no differences in glucose uptake between test and control muscles in either Akt-1 (A) or Akt-2 (B) rats. O/E, Overexpression.
ylation in muscles overexpressing Akt-1 but a more modest effect in those overexpressing Akt-2. These data are consistent with a model whereby muscle cell size is not determined by activation of a particular Akt isoform, but in the case of Akt-2 is potentially mediated through a distinct pathway to the Akt-mTOR-p70S6kinase pathway established for Akt-1 (3, 5). This possibility is supported by in vitro evidence that regulation of protein synthesis by Akt is isoform specific (32) and can occur independently of S6K, through a pathway involving mTOR-induced transcription of eukaryotic initiation factor 2B. It is conceivable that Akt-1 activation may be primarily coupled to p70S6kinase activation via mTOR, whereas Akt-2 preferentially targets eukaryotic initiation factor 2B. Furthermore, distinct pathways could be activated by insulin and IGF-I, with Akt-2 orchestrating the metabolic effects of insulin in muscle and Akt-1 mediating the growth-specific effects of IGF-I. Similar predictions have been made previously based upon work carried out in adipocytes in vitro (34).

We also observed a significant increase of around 60% in glycogen content at the conclusion of the 7-d experimental period in muscles overexpressing both Akt-1 and Akt-2. Whereas the simplest explanation of these results is that Akt-1 and -2 play similar roles in metabolism, this is inconsistent with previous data (7–9, 34), and several observations herein suggest that this is not the case. We observed a significant increase in acute glucose uptake and incorporation into both glycogen and lipids in muscle overexpressing Akt-2, but not Akt-1, and corroborated these results by demonstrating opposite effects of Akt-2 silencing. It has been suggested that glucose transport is rate limiting for many of the intracellular processes normally regulated by insulin in muscle (17, 35, 36). Therefore it is most likely that Akt-2 preferentially resulted in a significant increase in muscle glucose transport, which in turn resulted in increased flux of glucose into both glycogen and lipids. In contrast, the increase in glycogen mass observed in muscles overexpressing Akt-1 can be explained by an increase in glycolytic synthesis independent of glucose transport. The significant increase in GSK-3 phosphorylation detected in muscles overexpressing Akt-1, but not Akt-2, is consistent with this explanation.

Chronic activation of Akt has been proposed to generate a negative feedback signal via the mTOR-p70S6kinase pathway that results in reduced tyrosine phosphorylation and degradation of IRS-1 and, consequently, muscle insulin resistance (18–22). In this study chronic activation of both Akt-1 and Akt-2 resulted in increased phosphorylation of p70S6kinase and similar significant reductions in both IRS-1 phosphorylation and IRS-1 protein levels consistent with the proposed negative feedback effect, presumably mediated via increased proteasomal degradation. However, despite reductions in IRS-1 levels similar to those observed in naturally occurring glucose intolerance in humans (37) or those recorded in high-fat-fed (30) or chronic insulin-infused rats (19) and no compensation through up-regulation of IRS-2, we observed no consequent effect on muscle glucose uptake under insulin-stimulated conditions. It is also notable that we detected no enhancement in serine phosphorylation at residue 307 of IRS-1. This could be explained by the extremely rapid targeting of IRS-1 for degradation or by the kinase being principally responsible for Ser307 phosphorylation not being downstream of Akt.
Because the reduction in IRS-1 protein achieved as a result of ca-Akt overexpression was relatively modest, we attempted to confirm this result using an alternative model. To this end we administered shRNAs targeting IRS-1 in muscle in vivo and achieved a degree of silencing equal to or greater than that observed in more pronounced models of insulin resistance such as the Zucker fatty rat (31). However, there was still no difference in glucose disposal between treated and control muscles after a glucose load. These findings together imply that physiologically relevant reductions in IRS-1 protein are without consequence for muscle glucose disposal, suggesting that impairment in signaling through IRS-1 is unlikely to be a major mechanism mediating insulin resistance in this tissue.

In conclusion, we have identified alternative downstream signaling pathways triggered by activation of Akt-1 and Akt-2 in muscle in vivo. These isoforms have common effects to promote glycogen accumulation and hypertrophy of muscle, but we have shown that Akt-2 has a specific role in glucose disposal. In addition, chronic activation of the Akt-p70S6kinase pathway caused a negative feedback effect on IRS-1 expression. However, reductions in IRS-1 protein, triggered in this manner or through direct silencing of IRS-1, seem to have little relevance for muscle insulin sensitivity. These data have implications for the design of pharmaceuticals targeting the insulin signaling pathway as a treatment for insulin resistance.

MATERIALS AND METHODS

Vector Construction

The muscle-specific mammalian expression vector (EH114) and its GFP-expressing derivative (EH114-GFP) have been described previously (17). Vectors encoding constitutively active HA-tagged human Akt-1 and Akt-2 proteins incorporating the src myristoylation signal but lacking their pleckstrin homology domain were obtained from Dr. Morris Birnbaum (pLNCX-myrAkt-1 and pLNCX-myrAkt-2) (24). The Akt-2 construct was subcloned into the EcoRV/HindIII site of pBluescript SKI (Stratagene, La Jolla, CA) and then into the EcoRI/KpnI site of EH114. The Akt-1 construct was similarly subcloned via the SalI/Clal sites of the shuttle vector pOK12 (38) and then excised as a BamHI/EcoRV of EH114. Silencing constructs were generated by insertion of the shRNA cassette into either pU6.cass (39) as previously described. Two constructs were designed to express the following shRNAs targeting mouse IRS-1: pU6-GQ2, GCTGTCATCTAGTAGTACTTTGTGTAGGTAGGA-GTCCTGG(UU), the latter targeting two mRNA sequences. A control construct was subcloned into the pLNCX-myrAkt-1 and pLNCX-myrAkt-2 (40), incorporating administration of a bolus injection of 2-deoxy-D-2,6,3H]glucose and D-[U-14C]glucose (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). At the end of each study, rats were euthanized by iv injection of pentobarbitone sodium (Nembutal; Abbott Laboratories, Sydney, Australia), and their muscles were rapidly dissected and freeze-clamped using liquid nitrogen-cooled tongs. Plasma glucose tracer disappearance was used to calculate whole body glucose disposal (Rg). The area under the tracer disappearance 

In Vivo Electrotransfer

Preparation and injection of DNA and electrotransfer were carried out as previously described (17). Briefly, paired TC muscles were injected in rats with six spaced 50-μl aliquots and in mice with a single 30-μl aliquot of DNA prepared in endotoxin-free sterile saline (Qiagen Maxi/Mega-Prep kits, Doncaster, Victoria, Australia). For Akt isoform overexpression, right TC muscles from each animal were injected with ca-Akt-1 or ca-Akt-2 constructs whereas contralateral muscles were injected with EH114-GFP at 0.5 mg/ml. For Akt-2 or IRS-1 knockdown, 0.5 mg/ml each of pU6-GG3 and pU6-GG4 or pU6-GQ2 and pU6-GQ3, respectively, was injected into left TC muscles and 1 mg/ml of pU6-Luc was injected into the right TC muscles. Injections in rats were followed by delivery of one 800 V/cm 100-μsec electrical pulse and four 80 V/cm 100-msec pulses at 1 Hz sequentially and in mice by eight pulses of 200 V/cm and 20 msec at 1 Hz via tweezer electrodes attached to an ECM-830 electroporator (BTX Technologies, Inc., Holliston, MA).

Assessment of in Vivo Glucose Metabolism in Rats in the Basal and Insulin-Stimulated State

Conscious rats were studied after 5–7 h of fasting. Basal tracer uptake studies were conducted using a single jugular cannula connected to an infusion line when clamping was required. Rats were allowed to acclimatize to the study cage for 30–40 min. Hyperinsulinemic-euglycemic clamps of conscious rats and basal tracer uptake studies were performed as described (40), incorporating administration of a bolus injection of 2-deoxy-D-[2,6,3H]glucose and D-[U-14C]glucose (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). At the end of each study, rats were euthanized by iv injection of pentobarbitone sodium (Nembutal; Abbott Laboratories, Sydney, Australia), and their muscles were rapidly dissected and freeze-clamped using liquid nitrogen-cooled tongs. Plasma glucose tracer disappearance was used to calculate whole body glucose disposal (Rg). The area under the tracer disappearance
Combined IPGTT and 2-Deoxyglucose Tracer Uptake

A 7.4-MBq/ml solution of 2-deoxy-o-\([1-\text{\textsuperscript{14}}C]\)glucose (Amer sham Biosciences,) in 50% glucose/0.9% saline was prepared and injected at 2 g/kg body weight, approximately 0.37 MBq into 5- to 7-h starved mice (44). Blood was collected from the tail tip at 0, 15, 30, 45, 60, and 90 min post injection and blood glucose was measured immediately using an Accu-Check Advantage meter (Roche Diagnostics, Castle Hill, NSW, Australia). Blood radioactivity was subsequently determined at each time point by liquid scintillation counting, and the area under the curve was calculated. This, together with the disintegrations per minute (dpm) of phosphorylated 2-deoxy-o-\([1-\text{\textsuperscript{14}}C]\)glucose from individual muscles was used to calculate the clearance of tracer into each muscle.

Muscle Lysates, SDS-PAGE, and Immunoblotting

Protein expression and phosphorylation of molecules present in muscle were assessed by SDS-PAGE and quantification of Western blots of cell lysates. Whole-tissue lysates were prepared from dismembranated muscle (Mikro-dismembranator II, B. Braun Biotech, Melsungen, Germany) by 1100H pestle mixer (Astral Scientific, Gynsea, NSW, Australia) or homogenized in RIPA buffer ([65mmol/liter Tris; 150 mmol/liter NaCl; 0.5 mmol/liter sodium orthovanadate; 25 mmol/liter sucrose; 20 mmol/liter HEPES; 1 mmol/liter EDTA (pH 7.4); 5 mmol/liter EDTA (pH 7.4); 0.5 mmol/liter sodium deoxycholate; 0.1% (vol/vol) sodium dodecyl sulfate; 1% (vol/vol) sodium glycerol, containing 25 g/ml leupeptin; 10 g/ml aprotinin; 2 mmol/liter sodium orthovanadate; 1 mmol/liter sodium pyrophosphate; 1 mmol/liter ammonium molybdate; 10 mmol/liter NaF; and 1 mmol/liter polymethylsulfonfonyl fluoride) or HES buffer ([250 mmol/liter sucrose; 20 mmol/liter HEPES; 1 mmol/liter EDTA (pH 7.4); 0.5 mmol/liter sodium orthovanadate; 25 g/ml leupeptin; 10 g/ml aprotinin; 1 mmol/liter polymethylsulfonfonyl fluoride] followed by incubation for 90 min at 4 C and centrifugation for 10 min at 12,000 x g. Protein content of supernatants was quantified using the Bradford method (Protein Assay kit; Bio-Rad Laboratories, Regents Park, NSW, Australia), and aliquots containing 10-80 g protein were denatured in Laemmli buffer for 5 min at 95 C or 30 min at 37 C. Proteins were resolved by SDS-PAGE electrophoresis and electrotransferred as previously described (45). Immunoblotting and quantitation were also as previously described (45). Antibodies against GLUT-4 have been described previously (46). GFP antibody was purchased from Molecular Probes, Inc. (Sunnyvale, CA) pY612-IRS-1 from Biosource International (Camarillo, CA) IRS-2 (M-19) from Sigma-Aldrich (Castle Hill, NSW, Australia), Akt-1, Akt-2, total IRS-1, and acetyl coenzyme A decarboxylase from Upstate Cell Signaling Solutions (Waltham, MA), and all other antibodies were obtained from Cell Signaling Technology (Beverley, MA).

Muscle Sections and Immunohistochemistry

Transverse sections (10 mm) were cut from 10% formaldehyde-fixed paraffin-embedded test and control muscle portions removed from cohorts of ca-Akt-1 and ca-Akt-2-over-expressing rats. To determine the effects of each construct on fiber size, sections were dewaxed using xylene and ethanol, and antigen retrieval was undertaken in Tris-EDTA buffer at 95 C. Sections were blocked in 2% BSA in PBS for 30 min, incubated with 1:50 mouse anti-HA (Covance, North Ryde, NSW, Australia) and 1:200 rabbit antilaminin antibodies (Sigma-Aldrich) overnight in 0.1% BSA with antirabbit-Cy2 (green) plus antimouse-Cy3 (red) conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:200 for 1 h. Sections were examined using a Zeiss AxioVert 200M fluorescence microscope (North Ryde, NSW, Australia) under bright field, using appropriate filter sets (green; excitation, 450-490 nm; emission, 515-565 nm; red: excitation, 534-558 nm; emission, 575-640 nm) and analyzed using AxioVision Release 4.4 software. Fibers were delineated using basement membrane laminin fluorescence, and those fluorescing red were counted as positive for ca-Akt transfaction. To determine the percentage of type I fibers in each muscle, slides were baked for 2 h at 75 C, dewaxed, epitone retrieved in pH 9.0 1:10 EDTA Target Retrieval Solution (DAKO Corp., Carpenteria, CA) in a pressure cooker for 30 sec, and then processed in a Dako Autostainer using a 1:40 dilution of b slow heavy-chain myosin antibody (Alexis Biochemicals, Lausen, Switzerland), EnVisionTM Mouse secondary and diamino benzidine (DAB+) (DAKO Corp.) for visualization, followed by hematoxylin counterstaining. Sections were examined using a Leica DMRB microscope, DFC320 camera, and QWin software (Leica Microsystems, Gladesville, NSW, Australia). The number of stained fibers in four random x10 objective fields in each set of paired muscles were counted and expressed as a percentage of the total number of fibers.

Statistics

All data are quoted as mean ± SE. Comparisons between treated and control muscles were made using the paired Student’s t test or the Signed Rank test for non-normally distributed data. Comparisons between muscle fiber size in ca-Akt-1 and ca-Akt-2 electroeclated rats were made using two-way ANOVA followed by Holm-Sidak post hoc analysis with repeated measures for test vs. control muscles. Analyses were conducted using Sigma Stat version 3.00 (SPSS, Inc., Chicago, IL), with P < 0.05 regarded as significant.

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