0888-8809/07/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 21(1):215–228

doi: 10.1210/me.2006-0154

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

Mark E. Cleasby, Tracie A. Reinten, Gregory J. Cooney, David E. James, and Edward W. Kraegen

Diabetes and Obesity Program (M.E.C., T.A.R., G.J.C., D.E.J., E.W.K.), Garvan Institute of Medical Research; St. Vincent's Clinical School (G.J.C., E.W.K.), Faculty of Medicine, University of New South Wales; and School of Biotechnology and Biomolecular Sciences (D.E.J.), Faculty of Science, University of New South Wales, Sydney, Australia

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* electrotransfer 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)

THE ACTION OF insulin is mediated through the binding of insulin to its cognate receptor at the plasma membrane, triggering its autophosphorylation, tyrosine phosphorylation and binding of adaptor proteins [principally insulin receptor substrate-1 (IRS-1) in muscle], and hence activation of a series of intracellular signaling cascades. The phosphoinositide 3-kinase/Akt pathway has been shown to play a pivotal role in the regulation of glucose transport and glycogen synthesis in skeletal muscle cells (1, 2) and muscular hypertrophy *in vivo* (3). Downstream substrates of Akt involved in the regulation of these processes have been identified. These include glycogen synthase kinase-3, inactivation of which promotes glycogen synthesis (2), AS160, a Rab-GTPase-activating protein shown to be involved in the insulin-regulated trafficking of glucose transporter 4 (GLUT-4) to the cell surface (4), and p70S6kinase, which has been implicated in muscle hypertrophy (3, 5).

One of the complexities in studying the role of Akt in metabolism is that it has been shown to play an essential role in a number of key biological processes including cell growth and apoptosis (6). One hypothesis to account for the pleiotrophic effects of Akt is that different Akt isoforms may regulate discrete biological processes. Global knockout of both Akt isoforms results in growth retardation, although the effects of Akt-1 deficiency are more marked (7–9). In contrast, Akt-1 knockout mice demonstrate no impairment in glucose tolerance or insulin sensitivity (8) whereas Akt-2 deficient mice develop severe diabetes (7, 9), an effect that is mirrored by the effects of a naturally occurring human Akt-2 mutation (10). In ad-

First Published Online October 4, 2006

Abbreviations: ca, Constitutionally active; GFP, green fluorescent protein; GLUT, glucose transporter; GSK, glycogen synthase kinase; HA, hemagglutinin; IPGTT, ip glucose tolerance test; IRS-1, insulin receptor substrate 1; IVE, *in vivo* electrotransfer; mTOR, mammalian target of rapamycin; R_d, whole-body glucose disposal; shRNA, short hairpin RNA; TC, tibialis cranialis.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

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* electrotransfer (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 electrotransfer 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). We also compared the expression of the electrotransferred Akt constructs in muscle lysates on the same separate blot by immunoblotting with an anti-HA antibody to detect the hemagglutinin (HA) epitope present at the C terminus of both proteins and found that the relative degree of overexpression of the Akt-1 construct was expressed at a level 78% greater than the Akt-2 construct in its respective cohort of animals ($P = 0.004$). Expression of endogenous Akt-1 and Akt-2 was not significantly altered by either manipulation.

Akt Overexpression Causes Muscular Hypertrophy but Has No Effect on the Proportion of Type I Muscle Fibers

Transverse sections through test and control TC muscles were immunostained with anti-HA antibody to differentiate fibers expressing the ca-Akt constructs from nontransfected fibers. Consistent with previous results that used similar methodology (3), muscle fiber hypertrophy was induced by Akt overexpression. Ca-Akt-1 overexpression resulted in a 170% increase in cross-sectional area of transfected fibers (Fig. 2, A and D; $P < 0.001$) whereas ca-Akt-2 caused a 100% increase (Fig. 2, B and E; $P < 0.001$). The difference in fiber diameter between muscles overexpressing ca-Akt-1 and ca-Akt-2 was similar to the difference in overexpression of each isoform, suggesting that there is little isoform specificity in the control of muscle growth, at least as assessed in this experimental system.

Transverse sections from ca-Akt-1, ca-Akt-2, and control muscles were also immunostained for β slow myosin heavy chain to determine whether Akt overexpression resulted in altered muscle fiber type distribu-

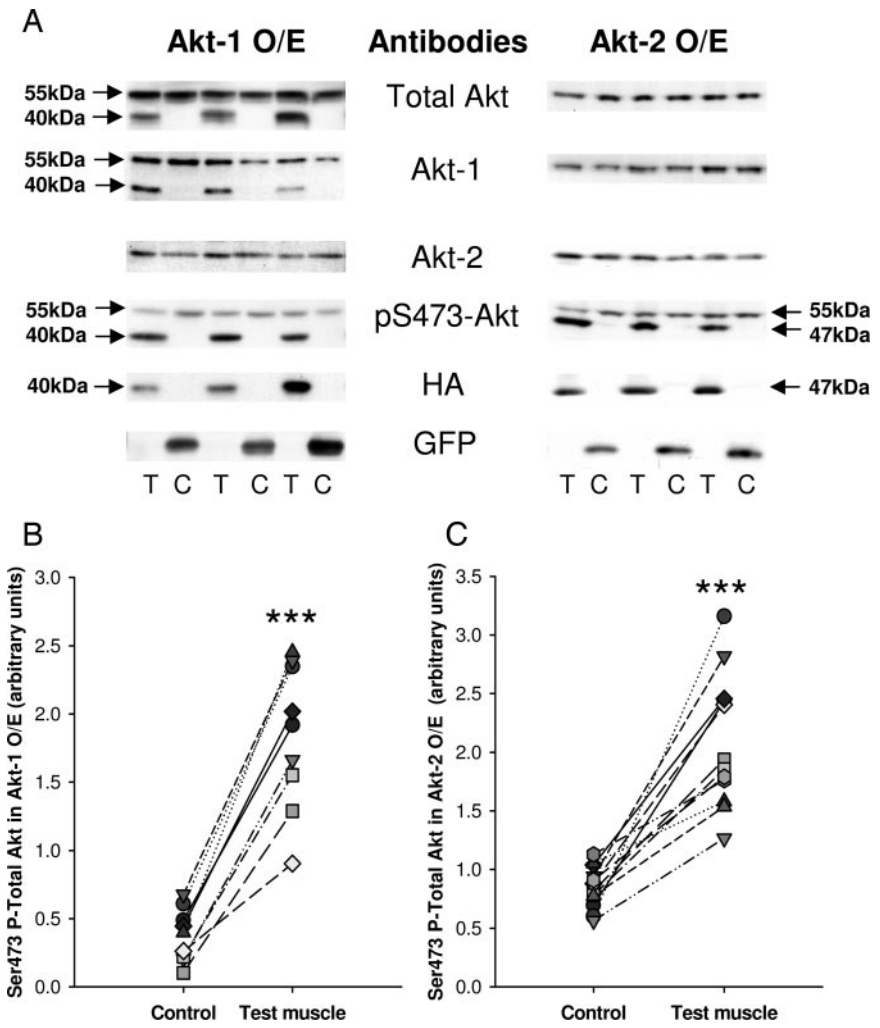

Fig. 1. Overexpression of Akt Isoforms in TC Muscle

Western blots of muscle lysates prepared from paired test (T) and control (C) muscles removed from rats 1 wk after electrotransfer demonstrating successful overexpression (O/E) of Akt (A). Blots *vs.* total Akt, Akt-1, Akt-2, total serine-473 phosphorylated Akt (pS473-Akt), HA tag, and GFP are shown. Bands at 55 kDa correspond to the endogenous Akt, whereas additional bands at approximately 40 and 47 kDa represent the exogenous constitutively active Akt species lacking the pleckstrin homology domain. The ca-Akt-2 species was not detected by the Akt-2 or total Akt antibodies due to disruption of the target epitopes. Graphs of total pS473-Akt in Akt-1 (B) and Akt-2 (C) electroporated rats showing a 490 and 157% increase, respectively, in total phosphorylation. ***, $P < 0.001$ *vs.* control muscle.

tion. 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 (R_d) 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

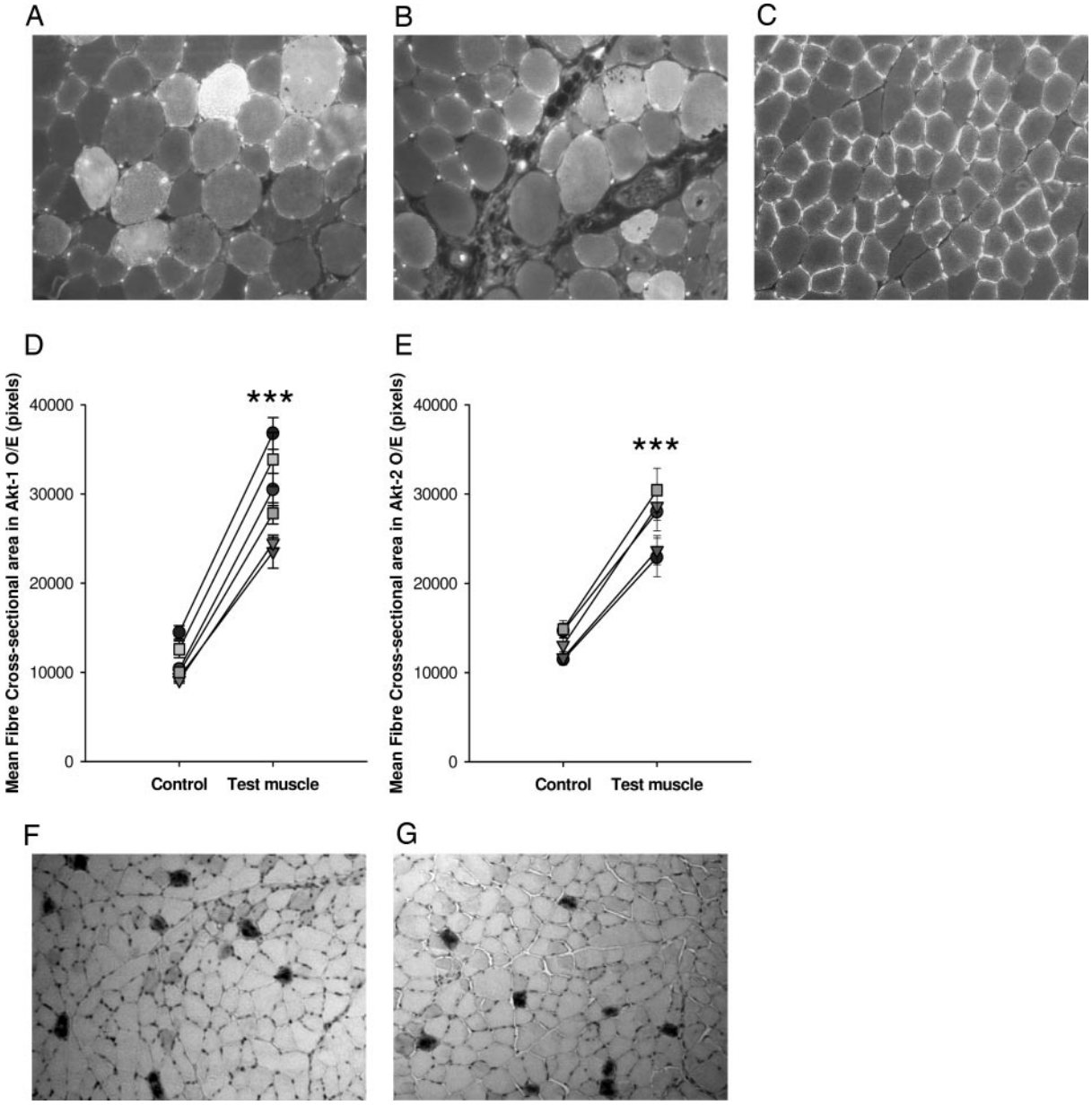

Fig. 2. Akt Overexpression Causes Muscle Fiber Hypertrophy but Has no Effect on the Proportion of Type I Muscle Fibers

Transverse sections through Akt-1 (A)- and Akt-2 (B)-overexpressing and control (C) muscles viewed under a ×20 objective. They are immunolabeled with laminin to delineate the fibers and an anti-HA antibody and demonstrate that transfected (*bright*) fibers show an increase in cross-sectional area. Graphs of mean fiber cross-sectional area in test *vs.* paired control muscles in Akt-1 (D) and Akt-2 (E) electroporated rats showing a mean 2.7- and 2.0-fold increase in test muscles, respectively. Transverse sections through paired Akt-1 (F) and control (G) muscles viewed under a ×10 objective after immunostaining for β slow myosin heavy chain. No difference in numbers of type I (*dark*) *vs.* type II (*light*) fibers are apparent. Similar results were obtained for Akt-2-overexpressing muscles. ***, $P < 0.001$ *vs.* control muscle. O/E, Overexpression.

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.

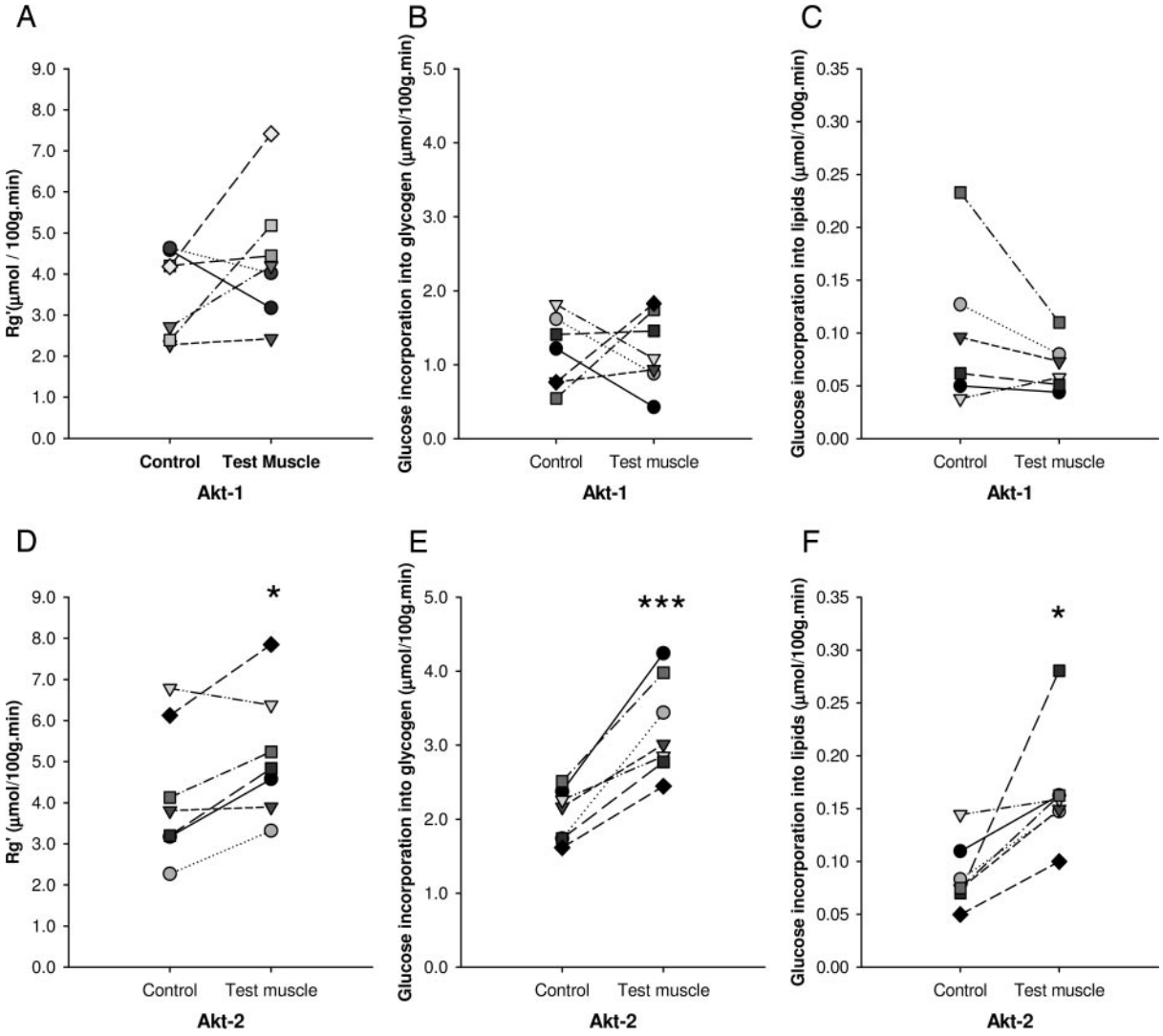

Fig. 3. Akt Overexpression Has Isoform-Specific Effects on Basal Glucose Uptake

Graphs showing that Akt-1 had no effect on glucose uptake (Rg′) (A), incorporation of glucose into glycogen (B), or incorporation of glucose into lipids (C) during study 1 wk after electrotransfer. In contrast, Akt-2 overexpression increased muscle glucose uptake by 36% (D), glucose incorporation into glycogen by 59% (E), and glucose incorporation into lipids by 108%. *, $P < 0.05$; ***, $P < 0.001$ *vs.* control muscle.

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-3 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-

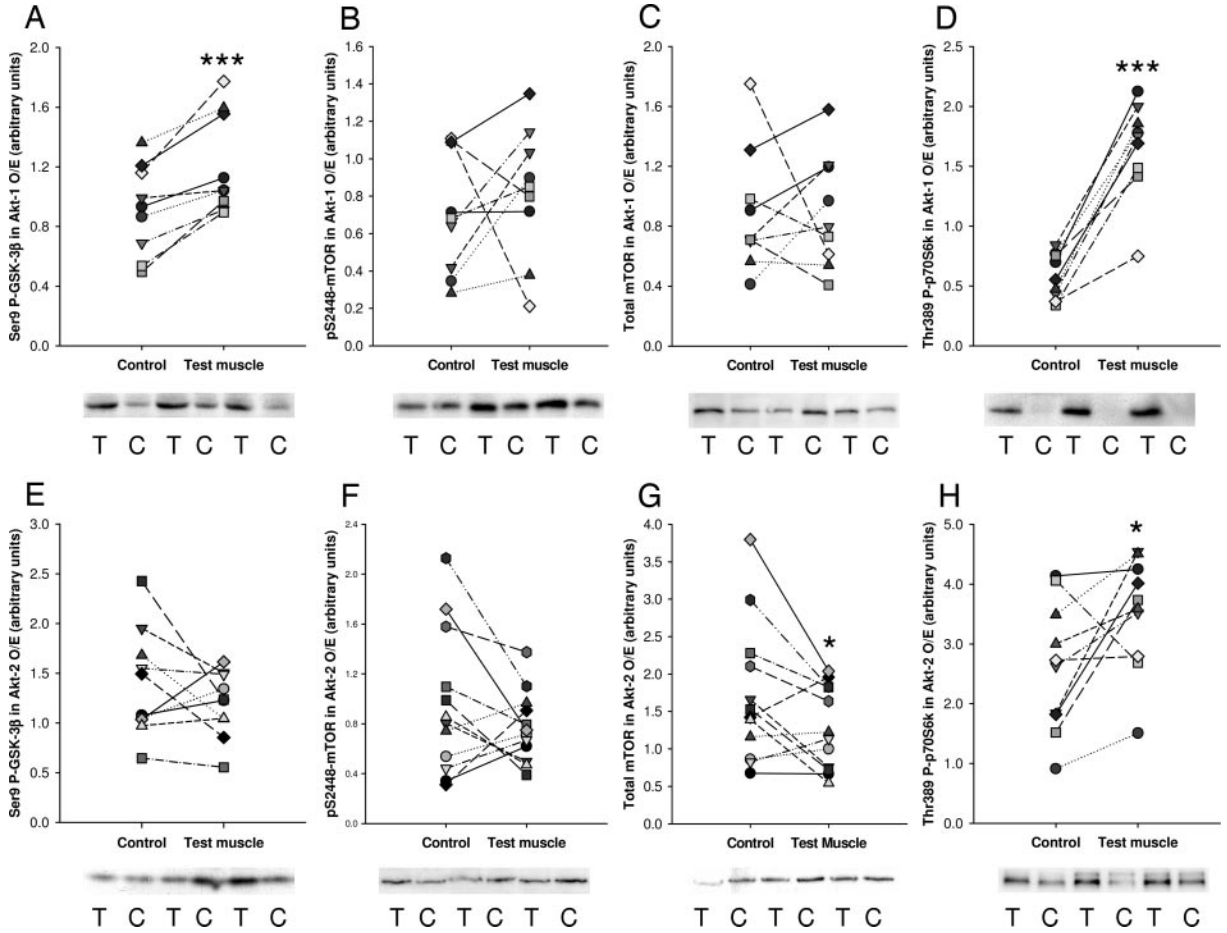

Fig. 4. Akt Overexpression Has Downstream Effects on GSK-3β, mTOR, and p70S6 Kinase in Muscle

Western blots and summary graphs showing effects of Akt overexpression on phosphorylation of GSK-3β [increased 38% in Akt-1 rats (A); unchanged in Akt-2 rats (E)], phosphorylation of mTOR [trend toward 40% increase in Akt-1 rats (B); no effect in Akt-2 rats (F)], protein expression of mTOR [no effect in Akt-1 rats (C); 17% decrease in Akt-2 rats (G)], and phosphorylation of p70 S6 kinase [increased 201% in Akt-1 rats (D); increased 53% in Akt-2 rats (H)]. *, $P < 0.05$; ***, $P < 0.001$ *vs.* control muscle. O/E, Overexpression; C, control; T, test.

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

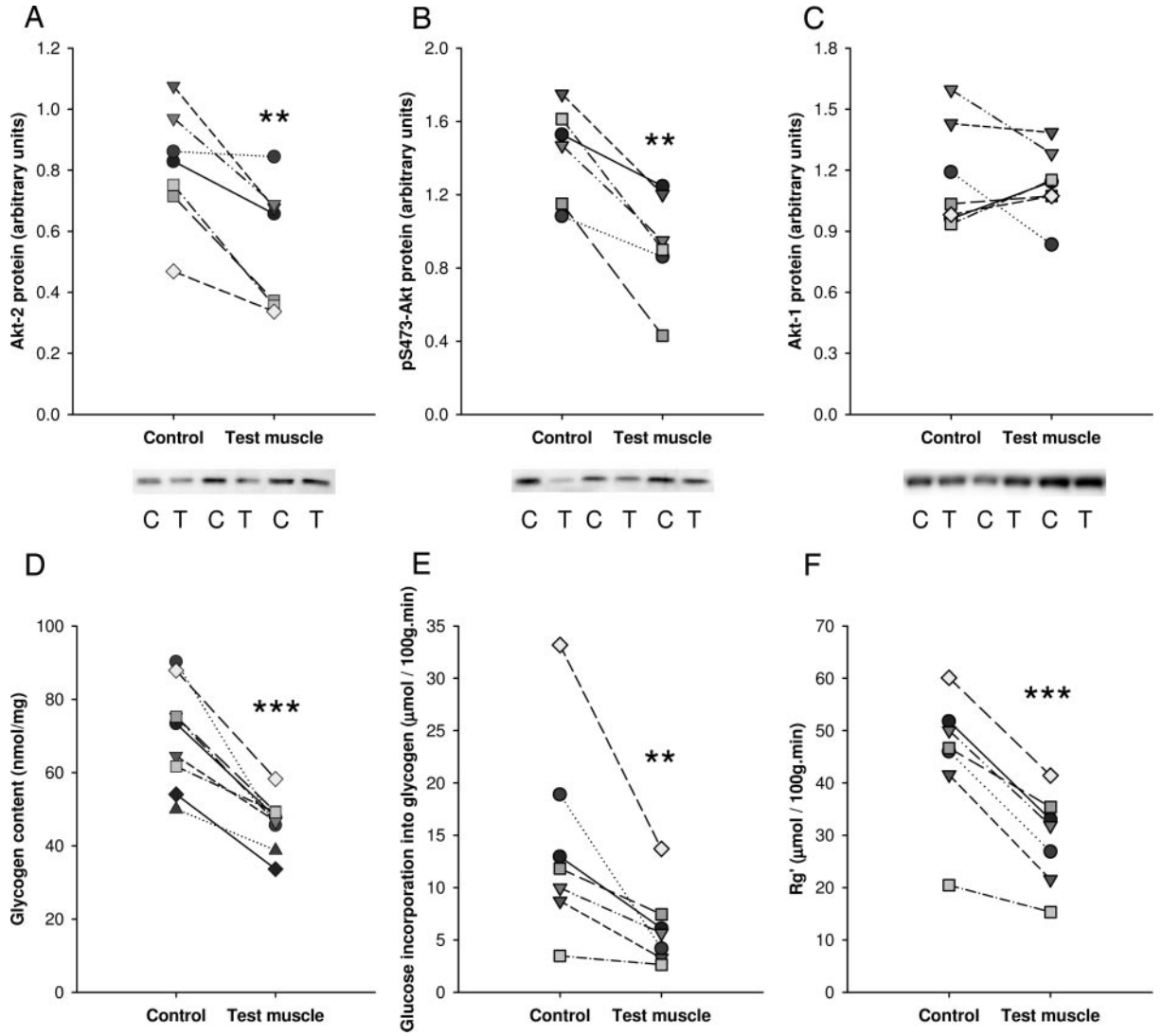

Fig. 5. Akt-2 Knockdown in Muscle Causes Local Reductions in Glycogen Storage and Insulin-Stimulated Glucose Uptake

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.

IRS-1 by a kinase pathway including mTOR and p70S6kinase. 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

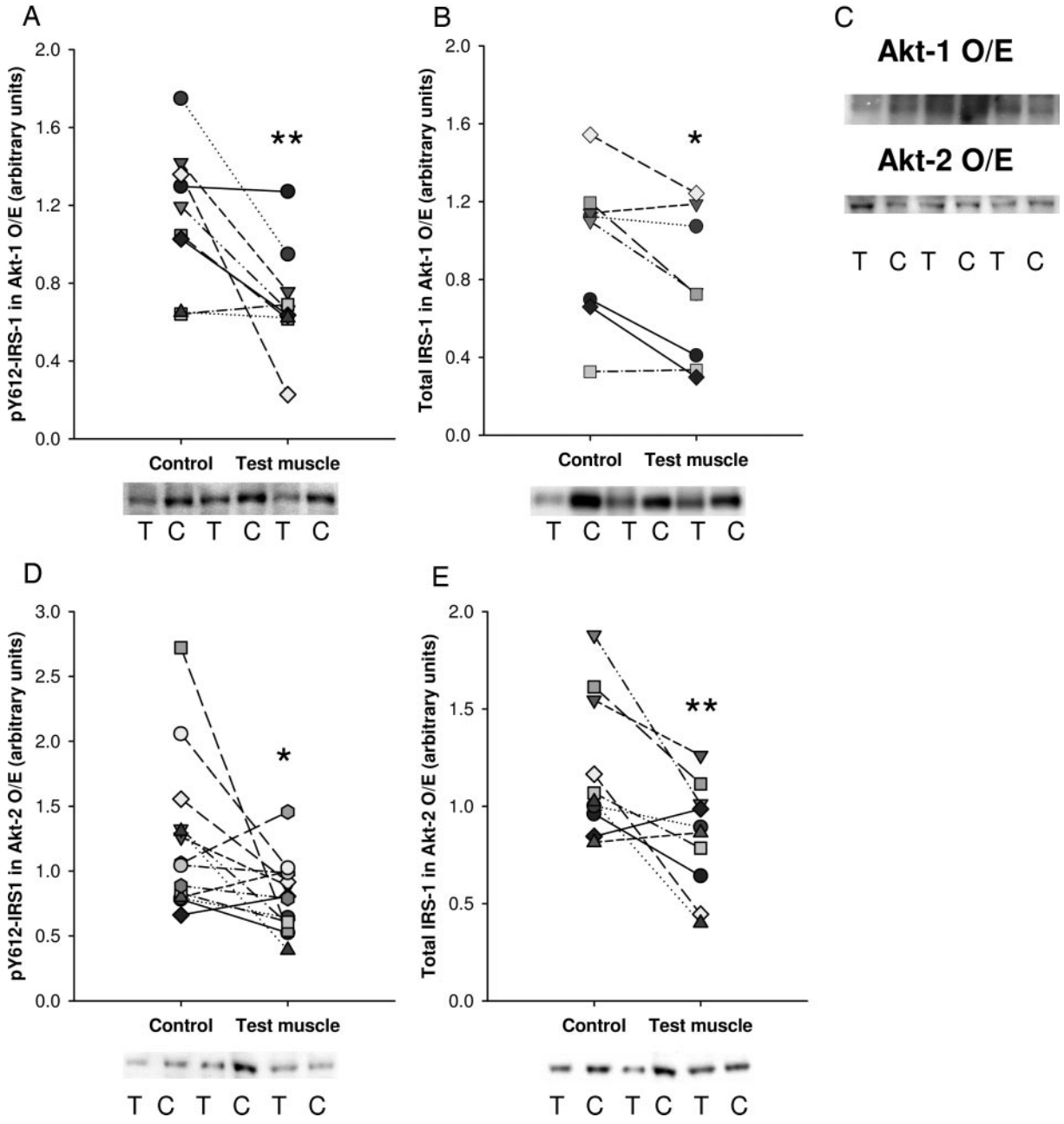

Fig. 6. Chronic Akt Activation Causes IRS-1 Down-Regulation in Muscle

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.

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

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 ± 2.6 mg/kg·min and 31.7 ± 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-

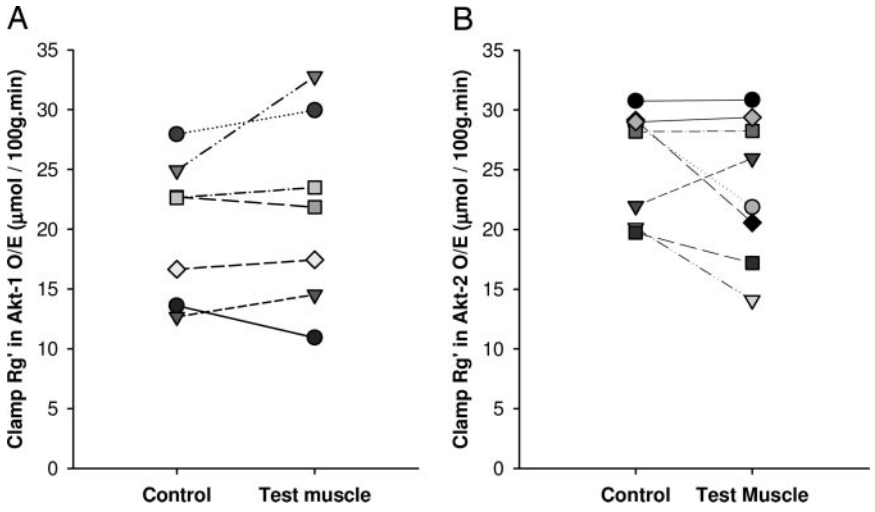

Fig. 7. Chronic Akt Activation Has No Effect on Insulin-Stimulated Glucose Uptake into Muscle

Graphs demonstrating that under hyperinsulinemic-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.

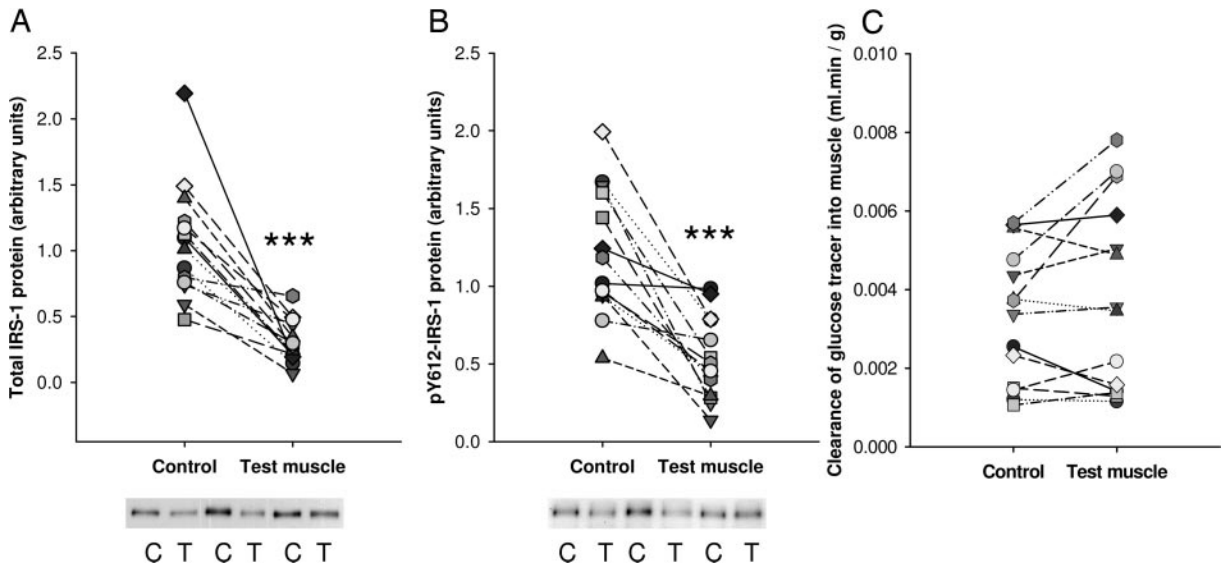

Fig. 8. shRNA-Mediated IRS-1 Silencing in Muscle Has No Effect on Local Glucose Clearance

Western blots and summary graphs demonstrating the effect of IRS-1 shRNA administration to TC muscles of mice 1 wk after IVE. Mean total IRS-1 protein (A) and pY-612-IRS-1 (B) were reduced by 68% and 53%, respectively. This gene silencing had no effect on clearance of glucose tracer into muscle as part of an IPGTT (C). ***, $P < 0.001$ *vs.* control muscle. C, Control; T, test.

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 2Bϵ (33). It is conceivable that Akt-1 activation may be primarily coupled to p70S6kinase activation via mTOR, whereas Akt-2 preferentially targets eukaryotic initiation factor 2Bϵ. 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 glycogen 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 *Eco*RV/*Hin*dIII site of pBluescript SK*II* (Stratagene, La Jolla, CA) and then into the *Eco*RI/*Kpn*I site of EH114. The Akt-1 construct was similarly subcloned via the *Sal*I/*Cla*I sites of the shuttle vector pOK12 (38) and then excised as a *Bam*HI/*Stu*I fragment which was ligated into *Bam*HI/*ECo*RV 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, GTCTGTCATCTAGTAGTACTTTGTGTAG-GTACTACTAGATGACAGAC(UU) and pU6-GQ3, GCACTGA-TGATGGCTATATGTTTGTGTAGCATATAGCCATCATCAGT-G(UU), and two targeting rat/mouse Akt-2: pU6-GG3, GCC-AGGACCACGAGCGCCTCTTTGTGTAGGAGGCGCTCGTG-GTCCTGG(UU) and pU6-GG4, GACTCCTCGGCAAGGGCA-CCTTAGCCAGGACCACGAGCGCCTCTTTGTGTAGGAGG-CGCTCGTGGTCCTGGTTGAGGTGCCCTTGCCGAGGAGT-(UU), the latter targeting two mRNA sequences. A control construct targeting luciferase mRNA (pU6-Luc) was also prepared, generating effectively nonsense shRNAs with sequence: GGCCTTTCACTACTCCTACTTTGTGTAGGTAGGA-GTAGTGAAAGGCC(UU). Molecular reagents were supplied by Promega Corp. [Annandale, New South Wales (NSW), Australia] and New England Biolabs (Genesearch Pty Ltd, Arundel, Queensland, Australia).

Animal Maintenance, Surgery, and Drug Treatment

Male Wistar rats (~150 g) or Swiss mice (~25 g) were obtained from the Animal Resources Centre (Perth, Australia) and acclimatized to their new surroundings for 1 wk. Animals were maintained at 22 ± 0.5 C under a 12-h day/12-h night cycle and were fed a standard chow diet (Norco, Kempsey, Australia) (18% fat, 33% protein, and 48% carbohydrate as a percentage of total dietary energy) *ad libitum*. Approximately 1 wk before study, the right and left jugular veins of rats designated to undergo clamp studies, or the right jugular alone for rats designated to undergo basal tracer uptake studies, were cannulated as previously described (40). Anesthesia was induced with 5% and maintained with 1–2% halothane in oxygen, the surgical site was irrigated with bupivacaine (0.5 mg/100 g) before closure, and 5 mg/kg ketoprofen was administered to provide postoperative analgesia. Rats were single housed and handled daily for the following week to minimize stress. Body weight was recorded daily, and only those rats that had fully recovered their presurgery weight were subsequently studied (n = 6–15 per group). All experimental procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

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 a sampling line between 0900 and 1000 h, whereas a second jugular cannula was also 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 or basal tracer uptake studies were performed as described (40), incorporating administration of a bolus injection of 2-deoxy-D-[2,6-^{3}H]glucose and D-[*U*-^{14}C]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 (R_d). The area under the tracer disappearance

curve of 2-deoxy-D-[2,6,-^{3}H]glucose together with the counts of phosphorylated [^{3}H]deoxyglucose from individual muscles were used to calculate insulin-stimulated glucose metabolic index (Rg′), an estimate of tissue glucose uptake (41). During clamps, plasma glucose was determined immediately using a glucose analyzer (model 2300; Yellow Springs Instrument Co., Yellow Springs, OH) with the remaining plasma being frozen and subsequently used for plasma insulin determination by RIA (Linco Research, Inc., St. Charles, MO). Tissue triglycerides were extracted (42) and measured using a Peridochrom Triglyceride GPO-PAP kit (Roche Clinical Laboratories, Indianapolis, IN). Muscle glycogen was analyzed as previously (43). Glucose incorporation into lipids and glycogen were determined from the D-[*U*-^{14}C]glucose tracer disappearance curve and counts of [^{14}C] in muscle as previously described (41).

Combined IPGTT and 2-Deoxyglucose Tracer Uptake

A 7.4-MBq/ml solution of 2-deoxy-D-[1-^{14}C]glucose (Amersham 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-D-[1-^{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 I100H pestle mixer (Astral Scientific, Gymea, NSW, Australia) or manual homogenization in RIPA buffer [65mmol/liter Tris; 150 mmol/liter NaCl; 5 mmol/liter EDTA (pH 7.4); 1% (vol/vol) Nonidet P-40 detergent; 0.5% (wt/vol) sodium deoxycholate; 0.1% (wt/vol) sodium dodecyl sulfate; 10% (vol/vol) glycerol, containing 25 μg/ml leupeptin; 10 μg/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 polymethylsulfonyl 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 μg/ml leupeptin; 10 μg/ml aprotinin; 1 mmol/liter polymethylsulfonyl fluoride) followed by incubation for 90 min at 4 C and centrifugation for 10 min at 12,000 $\times$ *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 μg 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 μm) were cut from 10% formaldehyde-fixed paraffin-embedded test and control muscle portions removed from cohorts of ca-Akt-1 and ca-Akt-2-overexpressing 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 transfection. To determine the percentage of type I fibers in each muscle, slides were baked for 2 h at 75 C, dewaxed, epitope 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 β slow heavy-chain myosin antibody (Alexis Biochemicals, Lausen, Switzerland), EnVisionTM+Mouse secondary and diaminobenzidine (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 $\times$10 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 $\pm$ 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 electroporated 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.

Acknowledgments

We thank Dr. Morris Birnbaum (Howard Hughes Research Institute, University of Pennsylvania, Philadelphia, PA) for the myristoylated Akt constructs; Dr. Edna Hardeman (Children's Medical Research Institute, Westmead, NSW, Australia) for the EH114 vector; and Dr. Michael Graham (Benitec, St. Lucia South, Queensland, Australia) for shRNA constructs. We thank Jane Radford (University of Sydney, Sydney, Australia), Quintin Lau (Garvan Institute), and Rebecca Brown (Garvan Institute) for expert technical assistance.

Received April 10, 2006. Accepted September 27, 2006.

Address all correspondence and requests for reprints to: Dr. Mark E. Cleasby, Diabetes and Obesity Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, New South Wales 2010, Australia. E-mail: M.Cleasby@garvan.org.au.

This work was supported by a project grant and fellowships (to G.J.C., D.E.J., and E.W.K.) from the National Health and Medical Research Council of Australia.

The authors have no conflicts of interest to declare.

REFERENCES

1. Hajduch E, Alessi DR, Hemmings BA, Hundal HS 1998 Constitutive activation of protein kinase B α by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. Diabetes 47:1006–1013
2. Ueki K, Yamamoto-Honda R, Kaburagi Y, Yamauchi T, Tobe K, Burgering BM, Coffer PJ, Komuro I, Akanuma Y, Yazaki Y, Kadowaki T 1998 Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. J Biol Chem 273: 5315–5322
3. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD 2001 Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 3:1014–1019
4. Bruss MD, Arias EB, Lienhard GE, Cartee GD 2005 Increased Phosphorylation of Akt Substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. Diabetes 54:41–50
5. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ 2001 Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 3:1009–1013
6. Yang ZZ, Tschopp O, Baudry A, Dummler B, Hynx D, Hemmings BA 2004 Physiological functions of protein kinase B/Akt. Biochem Soc Trans 32:350–354
7. Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, Brees DJ, Wicks JR, McNeish JD, Coleman KG 2003 Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB β. J Clin Invest 112:197–208
8. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ 2001 Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem 276:38349–38352
9. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, III, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ 2001 Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB β). Science 292:1728–1731
10. George S, Rochford JJ, Wolfrum C, Gray SL, Schinner S, Wilson JC, Soos MA, Murgatroyd PR, Williams RM, Acerini CL, Dunger DB, Barford D, Umpleby AM, Wareham NJ, Davies HA, Schafer AJ, Stoffel M, O'Rahilly S, Barroso I 2004 A family with severe insulin resistance and diabetes due to a mutation in AKT2. Science 304: 1325–1328
11. Tremblay F, Lavigne C, Jacques H, Marette A 2001 Defective insulin-induced GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both Akt/protein kinase B and atypical protein kinase C (ζ/λ) activities. Diabetes 50:1901–1910
12. Belfort R, Mandarino L, Kashyap S, Wirfel K, Pratipanawatr T, Berria R, DeFronzo RA, Cusi K 2005 Dose-response effect of elevated plasma free fatty acid on insulin signaling. Diabetes 54:1640–1648
13. Kim YB, Peroni OD, Franke TF, Kahn BB 2000 Divergent regulation of Akt1 and Akt2 isoforms in insulin target tissues of obese Zucker rats. Diabetes 49:847–856
14. Brozinick JT, Jr., Roberts BR, Dohm GL 2003 Defective signaling through Akt-2 and -3 but not Akt-1 in insulin-resistant human skeletal muscle: potential role in insulin resistance. Diabetes 52:935–941
15. Hill MM, Clark SF, Tucker DF, Birnbaum MJ, James DE, Macaulay SL 1999 A role for protein kinase Bβ/Akt2 in insulin-stimulated GLUT4 translocation in adipocytes. Mol Cell Biol 19:7771–7781
16. Liao W, Wang S, Han C, Zhang Y 2005 14–3-3 proteins regulate glycogen synthase 3β phosphorylation and inhibit cardiomyocyte hypertrophy. FEBS J 272: 1845–1854
17. Cleasby ME, Davey JR, Reinten TA, Graham MW, James DE, Kraegen EW, Cooney GJ 2005 Acute bidirectional manipulation of muscle glucose uptake by in vivo electrotransfer of constructs targeting glucose transporter genes. Diabetes 54:2702–2711
18. Carlson CJ, White MF, Rondinone CM 2004 Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation. Biochem Biophys Res Commun 316: 533–539
19. Ueno M, Carvalheira JB, Tambascia RC, Bezerra RM, Amaral ME, Carneiro EM, Folli F, Franchini KG, Saad MJ 2005 Regulation of insulin signalling by hyperinsulinaemia: role of IRS-1/2 serine phosphorylation and the mTOR/p70 S6K pathway. Diabetologia 48:506–518
20. Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, Donner DB 2001 A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc Natl Acad Sci USA 98:4640–4645
21. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G 2004 Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature 431:200–205
22. Tremblay F, Marette A 2001 Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem 276:38052–38060
23. Araki E, Lipes MA, Patti ME, Bruning JC, Haag III B, Johnson RS, Kahn CR 1994 Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature 372:186–190
24. Kohn AD, Takeuchi F, Roth RA 1996 Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. J Biol Chem 271:21920–21926
25. Armstrong RB, Phelps RO 1984 Muscle fiber type composition of the rat hindlimb. Am J Anat 171:259–272
26. Macaulay K, Blair AS, Hajduch E, Terashima T, Baba O, Sutherland C, Hundal HS 2005 Constitutive activation of GSK3 down regulates glycogen synthase abundance and glycogen deposition in rat skeletal muscle cells. J Biol Chem 280:9509–9518
27. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI 2002 Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J Biol Chem 277:50230–50236
28. Pirola L, Bonnafous S, Johnston AM, Chaussade C, Portis F, Van Obberghen E 2003 Phosphoinositide 3-kinase-mediated reduction of insulin receptor substrate-1/2 protein expression via different mechanisms contributes to the insulin-induced desensitization of its signaling pathways in L6 muscle cells. J Biol Chem 278:15641–15651
29. Haruta T, Uno T, Kawahara J, Takano A, Egawa K, Sharma PM, Olefsky JM, Kobayashi M 2000 A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol Endocrinol 14:783–794
30. Frangioudakis G, Ye JM, Cooney GJ 2005 Both saturated and n-6 polyunsaturated fat diets reduce phos-

phorylation of insulin receptor substrate-1 and protein kinase B in muscle during the initial stages of *in vivo* insulin stimulation. Endocrinology 146:5596–5603
31. Anai M, Funaki M, Ogihara T, Terasaki J, Inukai K, Katagiri H, Fukushima Y, Yazaki Y, Kikuchi M, Oka Y, Asano T 1998 Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. Diabetes 47:13–23
32. Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, Natesan S, Brugge JS 2005 Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J Cell Biol 171: 1023–1034
33. Kubica N, Bolster DR, Farrell PA, Kimball SR, Jefferson LS 2005 Resistance exercise increases muscle protein synthesis and translation of eukaryotic initiation factor 2Bϵ mRNA in a mammalian target of rapamycin-dependent manner. J Biol Chem 280:7570–7580
34. Bae SS, Han C, Mu J, Birnbaum MJ 2003 Isoform-specific regulation of insulin-dependent glucose uptake by Akt/PKB. J Biol Chem 278:49530–49536
35. Shulman GI 2000 Cellular mechanisms of insulin resistance. J Clin Invest 106:171–176
36. Ren JM, Marshall BA, Gulve EA, Gao J, Johnson DW, Holloszy JO, Mueckler M 1993 Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. J Biol Chem 268:16113–16115
37. Storgaard H, Song XM, Jensen CB, Madsbad S, Bjornholm M, Vaag A, Zierath JR 2001 Insulin signal transduction in skeletal muscle from glucose-intolerant relatives of type 2 diabetic patients. Diabetes [Erratum (2002) 51:552] 50:2770–2778
38. Vieira J, Messing J 1991 New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene 100:189–194
39. Rice RR, Muirhead AN, Harrison BT, Kassianos AJ, Sedlak PL, Maugeri NJ, Goss PJ, Davey JR, James DE, Graham MW 2005 Simple, robust strategies for generating ddRNAi constructs. Methods Enzymol 392:405–419
40. Clark PW, Jenkins AB, Kraegen EW 1990 Pentobarbital reduces basal liver glucose output and its insulin suppression in rats. Am J Physiol 258:E701–E707
41. Kraegen EW, James DE, Jenkins AB, Chisholm DJ 1985 Dose-response curves for in vivo insulin sensitivity in individual tissues in rats. Am J Physiol 248:E353–E362
42. Bligh EG, Dyer WJ 1959 A rapid method of total lipid extraction and purification. Can J Med Sci 37:911–917
43. Chan TM, Exton JH 1976 A rapid method for the determination of glycogen content and radioactivity in small quantities of tissue or isolated hepatocytes. Anal Biochem 71:96–105
44. Crosson SM, Khan A, Printen J, Pessin JE, Saltiel AR 2003 PTG gene deletion causes impaired glycogen synthesis and developmental insulin resistance. J Clin Invest 111:1423–1432
45. Cleasby ME, Dzamko N, Hegarty BD, Cooney GJ, Kraegen EW, Ye JM 2004 Metformin prevents the development of acute lipid-induced insulin resistance in the rat through altered hepatic signaling mechanisms. Diabetes 53:3258–3266
46. James DE, Strube M, Mueckler M 1989 Molecular cloning and characterization of an insulin-regulatable glucose transporter. Nature 338:83–87

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**