Disassociation of Insulin Action and Akt/FOXO Signaling in Skeletal Muscle of Older Akt Deficient Mice

Thomas H. Reynolds IV*, Erin Merrell, Nicholas Cinquino, Megan Gaugler, Lily Ng

Department of Health and Exercise Sciences, Skidmore College, Saratoga Springs, NY 12866

*Correspondence: Thomas H. Reynolds IV, Ph.D.

Department of Health and Exercise Sciences

Skidmore College

815 North Broadway

Saratoga Springs, NY 12866

Phone: (518) 580-8349

Fax: (518) 580-8356

Email: treynold@skidmore.edu

Key Words: Signal Transduction, Atrophy, Sarcopenia, Protein Kinase B, Glucose Metabolism

Copyright © 2012 by the American Physiological Society.
ABSTRACT

The purpose of the present study was to determine the effect of Akt gene ablation on Akt/FOXO signaling and atrogene expression. This was accomplished by studying wildtype (WT) and isoform specific Akt knockout (Akt1^−/− and Akt2^−/−) mice. The ability of insulin to promote Akt phosphorylation on Ser^473 was significantly lower in extensor digitorum longus (EDL) and soleus muscles from Akt1^−/− and Akt2^−/− mice compared to WT mice. Total Akt1 protein levels were significantly lower in EDL muscles of Akt2^−/− mice compared to WT mice, a process that appears to be post-transcriptionally regulated as Akt1 mRNA levels were unchanged. The loss of Akt1 protein in EDL muscles of Akt2^−/− mice does not appear to be due to insulin resistance as four months of a high fat diet failed to reduce Akt1 protein levels in muscles of WT mice. Although FOXO3a phosphorylation and atrogin-1 expression were unaltered in muscles of Akt1^−/− and Akt2^−/− mice, the expression of the atrogens, Bnip3 and gabarapl, were significantly elevated in muscles of both Akt1 and Akt2 knockout mice. Finally, the expression of striated activator of Rho signaling (STARS) was significantly increased in muscles of Akt2^−/− mice compared to Akt1^−/− and WT mice. Our results demonstrate that the ablation of Akt isoforms disassociates insulin action and Akt/FOXO signaling to atrogens.
INTRODUCTION

Akt (also called protein kinase B [PKB]) is a critical insulin signaling molecule that regulates muscle mass, in part, by suppressing protein degradation (4, 12). Akt appears to suppress protein degradation by orchestrating a decrease in the expression of genes necessary for ubiquitin-proteasome and autophagy-lysosomal dependent proteolysis (4, 14). One mechanism by which Akt reduces the expression of genes involved with protein degradation is the phosphorylation and subsequent nuclear exclusion of the forkhead box transcription factor FOXO. Recently, several FOXO-dependent genes involved with skeletal muscle proteolysis have been associated with muscle atrophy and coined “atrogenes” (3, 14, 22, 26, 27).

Akt exists in three different isoforms that are encoded by separate genes: Akt1, Akt2, and Akt3 (16, 17, 31). Through the generation of isoform-specific Akt knockout mice, distinct functions of the different Akt isoforms have been proposed. Akt1 is expressed in all tissues and appears to regulate growth as Akt1 knockout mice have smaller body size without any other noticeable phenotype (9). Akt2 is expressed in skeletal muscle, adipose tissue, and liver and appears to control glucose homeostasis as Akt2 knockout mice have impaired glucose tolerance and reduced insulin-stimulated glucose transport (8). Akt3 is primarily expressed in the brain and testes, but Akt3 knockout mice have no noticeable phenotype other than reduced brain size (11, 28). Therefore, it appears that Akt1 controls growth and Akt2 controls glucose metabolism; however, more recent studies suggest an overlapping of Akt functions between the Akt1 and Akt2 isoforms (6, 10, 30). For example, Dummler et al. (6, 10, 30) demonstrated that mice that only express Akt1 (Akt2/3 double knockouts) are growth deficient while other studies have shown that Akt1 null mice have enhanced insulin sensitivity (6, 10, 30).

Although Akt is known to regulate muscle mass by controlling FOXO-dependent atrogene expression, the Akt isoform that controls this process is not established. Therefore, the
first goal of this study was to determine the effect of isoform specific Akt gene ablation on atrogene expression. The effect of isoform specific Akt ablation on Akt/FOXO signaling in muscles comprised of distinctly different fiber types is unknown. In this regard, the second goal of our study was to determine the effect of isoform specific Akt gene ablation on Akt/FOXO signaling in the soleus, a muscle that expresses predominantly type I oxidative fibers, and the extensor digitorum longus, a muscle that expresses predominantly type II glycolytic fibers. To accomplish these two goals, we studied wildtype (WT) and isoform specific Akt knockout (Akt1−/− and Akt2−/−) mice at the age of 18 months old. Eighteen-month-old WT and Akt knockout mice were utilized based on the hypothesis that aging would accentuate specific actions of the respective Akt isoforms. Our results indicate that Akt1 and Akt2 ablation does not alter atrogin-1 expression, but increases Bnip3 and gabarap1, two autophagy related atrogene (3, 14, 22, 26, 27). Furthermore, we discovered that Akt2 knockout mice do not express Akt1 protein in EDL muscles, a factor that likely contributes to the insulin resistance observed in Akt2 null mice.

METHODS

Animals

Wildtype C57B6 male mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Akt1−/− and Akt2−/− mice (>10th generation C57B6 backcross) were a generous gift from Dr. Morris J. Birnbaum (University of Pennsylvania, Philadelphia, PA). Upon arrival to the animal facility at Skidmore College, all mice (~3 months old) were housed individually with cage enrichment nest-lets and fed ad libitum chow and water. Wildtype, Akt1−/−, and Akt2−/− mice were housed in the facility until the age of 18 months before experiments were conducted, unless stated otherwise. For dietary induced insulin resistance experiments, C57B6 male mice (~2 months old) were fed a high fat diet (HFD) or a normal chow diet (CON) for four months. All
animal care and surgery were conducted in accordance with the National Research Council's Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996). All experimental protocols were approved by Skidmore College's Institutional Animal Care and Use Committee.

In Vivo Insulin Action

To assess the effect of Akt ablation on in vivo insulin action, mice were subjected to an insulin-assisted glucose tolerance (IAGT) test following a 6-hour fast. Mice were simultaneously injected with glucose (2 g/Kg body weight) and insulin (18 U/Kg body weight). Glucose was measured by a glucometer in blood collected via the tail vein at 0, 15, 30, 45, and 60 min following the glucose/insulin injection. We have previously shown that the IAGT test detects insulin resistance as well as an insulin tolerance test in mice fed a high fat diet but avoids the severe hypoglycemia that is typically observed during and after insulin tolerance testing (25).

Surgical Procedures and In Vitro Muscle Incubations

For harvesting tissues, mice were anesthetized with a 1:1:1 mixture of promace, ketamine hydrochloride, and xylazine by an intraperitoneal injection (0.015 ml/10 g body weight). EDL and soleus muscles were isolated intact for in vitro incubation, and quadriceps and tibialis anterior (TA) muscles were rapidly dissected, frozen in liquid nitrogen, and stored at –80°C until analysis. Isolated EDL and soleus muscles were transferred to 25-ml Erlenmeyer flasks (1 muscle/flask) containing 2 ml of oxygenated Krebs Henseleit buffer (0.160 M NaCl, 0.0046 M KCl, 0.0012 M KH₂PO₄, 0.0025 M NaHCO₃, 0.0025 M CaCl₂, and 0.0012 M MgSO₄), 5 mM glucose, 0.75 mU/ml insulin and incubated for 30 min at 37°C with shaking. All flasks were pre-gassed with 95% O₂ and 5% CO₂ for 3 minutes immediately prior to the incubations. Following the 30 min incubation, muscles were frozen in liquid N₂ and stored at -80°C.
**Preparation of Muscle Extracts**

The frozen EDL, soleus, and TA muscles were homogenized on ice using a motor-driven tissue grinder (Teflon-glass) in RIPA Buffer (Sigma Chemical, Inc.) (1 ml buffer: 0.1 g muscle weight) containing protease and phosphatase inhibitor cocktails (Halt Protease/Halt Phosphatase, Thermo Fisher). The homogenates were rotated at 4 °C for 1 h and then centrifuged at 9,000 x g for 30 min at 4 °C. The protein concentrations of the supernatants were determined by the BCA method (Pierce, Inc). The remaining skeletal muscle extract was utilized for electrophoretic analysis and immunoblotting experiments.

**Electrophoretic Analyses and Immunoblotting**

Skeletal muscle extracts and molecular weight standards (Bio-Rad, Hercules, CA and Magic Mark, Invitrogen) were subjected to SDS-PAGE. The proteins were then electrophoretically transferred to Immobilon membranes and immunoblotted with the phospho-specific Akt antibodies, pThr\(^{308}\) Akt and pSer\(^{473}\) Akt that recognize Akt when phosphorylated on Thr\(^{308}\) and Ser\(^{473}\), respectively. The phosphorylation of Thr\(^{308}\) and Ser\(^{473}\) is necessary for Akt activity (1). The phosphorylation of FOXO3a was assessed by a phospho–specific antibody that recognizes FOXO3a only when phosphorylated on Thr\(^{32}\), a site phosphorylated by Akt (29). After washing the membranes, the light generated by the alkaline phosphatase conjugated secondary antibody and CDP-Star reagent was detected using a digital imaging system (UVP, Upland, CA). Phospho-specific immunoblots were stripped and re-probed with the Akt1 and Akt2 antibodies. An atrogin-1 antibody was used to assess atrogin-1 protein levels. To account for gel loading differences, all immunoblots were stripped and re-probed with an α-tubulin antibody. Relative signal intensities of the Akt1, Akt2, pThr\(^{308}\) Akt, pSer\(^{473}\) Akt, pThr\(^{32}\) FOXO3a, atrogin-1, and α-tubulin bands were determined by using the Total Lab software (Nonlinear,
Inc., Durham, NC). All data were normalized to α-tubulin and expressed as a percentage of wildtype controls. The phospho-specific Akt and FOXO3a antibodies and the α-tubulin antibody were from Cell Signaling Technology (Beverly, MA), the atrogin-1 antibody was from EMC Biosciences (Versailles, KY) and the Akt1 and Akt2 antibodies were a generous gift from Dr. Morris J. Birnbaum (University of Pennsylvania, Philadelphia, PA).

**Skeletal Muscle RNA Extraction and Real Time Quantitative PCR**

Frozen quadriceps muscles from mice were manually ground with a porcelain mortar and pestle chilled in liquid N$_2$. Total RNA was extracted from powdered muscle using an RNA extraction kit from Qiagen (RNeasy Fibrous Tissue Kit) and quantified by measuring absorbance at 260 nm using a spectrophotometer (Beckman Coulter, Brea, CA). Frozen EDL muscles were homogenized in a glass tube by a motor-driven homogenizer and total RNA was extracted from the resultant homogenate and quantified in an identical manner as quadriceps muscles. A 1 ug aliquot of total RNA was reverse transcribed using the RETROscript kit from Ambion (Austin, TX). The resultant cDNA (20 ng cDNA/sample in triplicate) was then subjected to quantitative polymerase chain reaction (qPCR) using standard target specific TaqMan gene expression assays and a real time PCR system (StepOne Plus Real-Time PCR System, Applied Biosystems, Foster City, CA). Relative quantitation of amplified cDNA targets were determined by the ΔΔCT method using StepOne v2.1 software (Applied Biosystems). The following TaqMan gene expression assays were used: Akt1 (Assay ID: Mm01331626_m1), atrogin-1 (Assay ID: Mm01207878_m1), Bnip3 (Assay ID: Mm00833810_g1), LC3 (Assay ID: Mm00458724_m1), gabarapl (Assay ID: Mm00457880_m1), and striated activator of Rho signaling (STARS) (Assay ID: Mm00615375_m1).
Statistical Analysis

To detect statistical significance for all dependent variables, a one-way analysis of variance (ANOVA) was utilized. Following a significant F ratio, Fisher’s LSD post-hoc test was used to locate statistical differences between groups. Data are expressed as means ± SEM, and the level of statistical significance was set at p ≤ 0.05.

RESULTS

Physical Characteristics of WT, Akt1−/−, and Akt2−/− Mice

The effect of isoform specific Akt ablation on body weight, adiposity, and muscle mass has been previously described in young mice (8, 9, 15), but these variables have not been studied in older Akt knockout mice. As shown in table 1, body weight is significantly greater in WT mice compared to Akt1−/− or Akt2−/− mice and Akt1−/− mice weighed significantly less than aged-matched Akt2−/− mice. The differences in body weight among wildtype and Akt knockout mice can be partially explained by the reduction in muscle mass and adiposity. Table 1 demonstrates that EDL muscle mass is significantly lower in Akt1−/− and Akt2−/− mice compared to WT mice. Soleus muscle mass of Akt1−/− mice were significantly less than WT mice. However, the effects of Akt gene ablation on muscle mass appear to be due, in part, to a reduction in total body mass as the muscle mass/body weight ratio indexes were not significantly different across genotypes (table 1). With regards to adiposity, WT mice had significantly greater epididymal adipose tissue mass/body weight ratio than the Akt1−/− and Akt2−/− mice, indicating that differences in body weight are related to adipose tissue mass.

In Vivo and In Vitro Insulin Action

In order to assess the effects of isoform specific Akt ablation on in vivo insulin action, we conducted IAGT tests. In the fasted basal state, blood glucose values were similar among the groups (WT: 109.8±4.7 vs. Akt1−/−: 97.8±4.0 vs. Akt2−/−: 95.0±5.2). Blood glucose levels of Akt1−/−
mice were significantly lower than WT and Akt2−/− mice at 30, 45, and 60 min following a simultaneous injection of glucose and insulin, indicating enhanced insulin action in Akt1 null mice (Figure 1A). Blood glucose levels in Akt2−/− mice were significantly higher than age-matched WT mice, demonstrating profound insulin resistance (Figure 1A). Figure 1B shows that the incremental area under the curve (AUC) is significantly lower in WT and Akt1−/− mice compared to Akt2−/− mice, however, there was only a trend for differences in incremental AUC between WT and Akt1−/− (P = 0.10). These findings indicate that the loss of Akt1 appears to promote insulin sensitivity while the loss of Akt2 leads to severe insulin resistance.

To assess in vitro insulin action, we incubated intact EDL and soleus muscle with insulin and measured Akt phosphorylation on Thr308 and Ser473 and FOXO3a phosphorylation on Thr32. Figures 2A and 3A show representative immunoblots containing EDL and soleus muscle extracts, respectively, that were prepared with a Thr308 or a pSer473 phospho-specific antibody. In EDL muscles from Akt2−/− mice, pThr308 and pSer473 immunoreactivity was significantly lower than in muscles from Akt1−/− mice (Figures 2B and 2C). In soleus muscles, pSer473 phosphorylation was significantly lower in Akt2−/− mice compared to Akt1−/− and WT mice (Figure 3C). A 1 x 3 ANOVA only revealed a trend for a decrease in pThr308 phosphorylation in soleus muscle (P = 0.09), although a T-test showed significantly lower pThr308 immunoreactivity in soleus muscle from Akt2−/− compared to Akt1−/− mice (Figure 3B). No significant differences in pThr308 or pSer473 immunoreactivity were observed in EDL or soleus muscles from Akt1−/− mice compared to WT mice. Despite the reduced ability for insulin to promote Thr308 and Ser473 phosphorylation in EDL and soleus muscles from Akt2−/− mice, FOXO3a phosphorylation on Thr32 was unchanged (Figure 4), indicating that sufficient residual Akt activity maintained FOXO3a phosphorylation. It is important to note that we were technically unable to quantify total FOXO3a levels, therefore, we cannot rule out the possibility that changes in total amounts of FOXO3a may have influenced our Thr32 phosphorylation results.
Effects of Akt Ablation on Akt Expression

We also assessed the effects of Akt ablation on Akt1 and Akt2 expression in EDL and soleus muscles. Figure 2A shows representative immunoblots containing EDL muscle extracts that were prepared with an Akt1 or an Akt2 antibody. As expected, EDL muscles from Akt1−/− and Akt2−/− mice had significantly less Akt1 and Akt2 expression, respectively, compared to EDL muscles from WT mice (Figures 2D and 2E). Surprisingly, in EDL muscles of Akt2−/− mice we observed a significant reduction in Akt1 expression (Figure 2D). Figure 3A shows representative immunoblots containing soleus muscle extracts that were prepared with an Akt1 or an Akt2 antibody. As expected, the soleus muscles from Akt1−/− and Akt2−/− mice had significantly less Akt1 and Akt2 expression, respectively, compared to soleus muscles from aged-matched WT mice (Figures 3D and 3E). Unlike EDL muscles of Akt2−/− mice, there was no change in Akt1 expression in soleus muscles from Akt2−/− mice (Figure 3D).

We also conducted experiments to determine if the loss of Akt1 expression in EDL muscles of Akt2−/− mice was regulated at the transcriptional level. Figure 5A shows that Akt1 mRNA levels are not reduced in quadriceps muscles of Akt2−/− mice compared to WT mice. Unlike the EDL muscle, the quadriceps muscle contains a mix of type I and type II fibers; therefore, we assessed Akt1 mRNA levels in EDL muscles of younger Akt2−/− mice and demonstrated no differences in Akt1 mRNA compared to age-matched wild type mice (Figure 5B). Because Akt1 mRNA of EDL muscles was assessed in young Akt2−/− mice (4 months old), rather than 18 month-old mice, we wanted to confirm that Akt1 protein was also reduced. As expected Akt1 protein levels were lower in EDL muscles of 4-month old Akt2−/− mice compared to age-matched wildtype mice (Figure 5C). These observations provide evidence indicating that
the loss of Akt1 expression in EDL muscles of Akt2−/− mice is not mediated by reductions in Akt1 gene transcription.

**Akt1 Expression in Diet-Induced Insulin Resistance**

It is possible that the loss of Akt1 in EDL muscles of Akt2−/− mice was due, in part, to the severe insulin resistance observed in these mice. In this regard, we conducted Akt1 immunoblotting experiments in muscles from WT mice fed a HFD or CON diet for four months. Figure 6 shows that diet-induced insulin resistance did not alter Akt1 immunoreactivity in tibialis anterior muscle. The tibialis anterior muscle contains a similar proportion of type I and II fibers as the EDL muscle and, therefore, serves as a good surrogate since we did not have EDL muscles from mice fed a HFD.

**Effects of Akt Ablation on Atrogate Expression**

Because Akt plays an important role in the regulation of skeletal muscle size, we examined the expression of several muscle atrophy-related genes (atrogenes). Atrogin-1 is an E3 ubiquitin ligase whose expression is suppressed by Akt activity and the subsequent nuclear exclusion of the transcription factor FOXO. We observed no significant changes in atrogin-1 mRNA or protein levels in quadriceps, soleus, and EDL muscles of Akt1−/− or Akt2−/− mice (Figures 7A and 8). These results are surprising when considering the observation that Akt2−/− mice have a substantial decrease in Ser473 phosphorylation. However, as shown in Figure 4, FOXO3a phosphorylation on Thr32 is identical in EDL and soleus muscles from Akt1−/−, Akt2−/−, and WT mice indicating that the subcellular location of FOXO3a was likely unchanged with Akt ablation; however, we cannot rule out the possibility that total FOXO3a levels might have influenced our Thr32 phosphorylation data. Although FOXO3a phosphorylation appears to be unchanged, we observed a significant increase in Bnip3 and gabarapl, two autophagic related genes that are up regulated in multiple models of muscle atrophy (18, 22), in muscles from
Akt1−/− and Akt2−/− mice. However, expression of LC3 did not change across genotypes (Figure 7D).

**Effect of Akt Ablation on STARS Expression**

STARS is an actin binding protein that promotes actin polymerization and stimulates serum response factor (SRF) gene transcription. Increases in STARS expression is associated with pressure overload induced hypertrophy in the mouse heart (20) and skeletal muscle hypertrophy in humans following resistance training (21). To determine a potential role for Akt in STARS regulation, we assessed STARS expression in muscles of our Akt knockout mice. Interestingly, STARS expression increased significantly in muscles from Akt2−/− mice, but not Akt1−/− mice (Figure 9).

**DISCUSSION**

The Akt signaling pathway regulates both insulin-stimulated glucose transport and skeletal muscle mass (4, 5, 31). Akt1 and Akt2 isoforms have been suggested to have distinct functions. Akt1 knockout mice have impaired growth and development but normal glucose metabolism; whereas Akt2 knockout mice experience normal growth and development but have decreases in whole body and skeletal muscle insulin sensitivity (8, 9). In order to accentuate potential isoform specific effects of Akt1 and Akt2 on insulin action, Akt/FOXO signaling, and atrogene expression, the present investigation studied WT, Akt1−/−, and Akt2−/− mice at the age of 18 months.

We demonstrate that Akt2−/− mice are severely insulin resistant. Similar to other investigations, the insulin resistance observed in Akt2 null mice is a unique phenotype that is not associated with obesity (7, 8, 13). Since muscle is the primary site for insulin-stimulated glucose disposal, it is reasonable to hypothesize that Akt2−/− mice have a defect in insulin signal transduction in this important metabolically active tissue. This idea is supported by a significant
decline in insulin-stimulated glucose transport in EDL muscles from young Akt2 null mice; however, soleus muscles responded to insulin as well as wildtype mice (8). With this in mind, the present study examined Akt phosphorylation (pThr\textsuperscript{308} and pSer\textsuperscript{473}) and isoform specific Akt expression in EDL and soleus muscles from Akt2\textsuperscript{-/-} mice and WT mice. In EDL muscles from Akt2\textsuperscript{-/-} mice, we demonstrated a significant reduction in Akt1 expression that was so dramatic that levels were similar to the ablated Akt2 isoform (Figure 2C). The loss of Akt1 expression in EDL muscles of Akt2 null mice appears to create a double knockout and likely accounts for the large decline in pSer\textsuperscript{473} phosphorylation in this tissue (Figure 2B). However, in soleus muscles from Akt2\textsuperscript{-/-} mice, Akt1 expression is similar to age-matched controls (Figure 3C). Therefore, the loss of Akt1 in EDL muscle of Akt2\textsuperscript{-/-} mice indicates that the deletion of Akt2 disrupts Akt1 expression in a muscle comprised of predominantly type II glycolytic fibers, but not in a muscle comprised predominantly of type I oxidative fibers. Importantly, this observation may explain why Cho et al. (8) observed a decline in insulin-stimulated glucose transport in EDL muscles but not soleus muscles of Akt2\textsuperscript{-/-} mice.

The present study demonstrates that the loss of Akt1 expression in EDL muscles of Akt2\textsuperscript{-/-} mice is unlikely due to a defect in the transcription of the Akt1 gene. We have shown that Akt1 mRNA levels in quadriceps of Akt2\textsuperscript{-/-} mice are actually slightly higher than age-matched WT mice (Figure 5A). Because the quadriceps muscle contains a mixture of type I and type II muscle fibers, we repeated these experiments in EDL muscle of young Akt2\textsuperscript{-/-} mice and demonstrated that Akt1 mRNA was not altered (Figure 5B). Since young Akt2\textsuperscript{-/-} mice (4 months old) were utilized to assess Akt1 mRNA levels in EDL muscles, we wanted to confirm that Akt1 protein levels declined in a similar fashion as in older Akt2\textsuperscript{-/-} mice (18 months old). Therefore, we conducted immunoblotting experiments with EDL extracts prepared from young Akt2\textsuperscript{-/-} mice and observed a significant decline in Akt1 protein levels (Figure 5C), demonstrating that the levels of Akt1 protein in EDL muscles of Akt2 null mice are regulated at the post-transcriptional
level. It is important to note that the magnitude of the decline in Akt1 protein levels in EDL muscles from young Akt2\textsuperscript{+/−} mice was not as robust as in the older Akt2\textsuperscript{+/−} mice, indicating that advancing age promotes the loss of Akt1 protein content when Akt2 is absent (compare Figure 2C with Figure 5C).

Because Akt2 gene ablation produces severe insulin resistance, it is possible that the decline in Akt1 protein expression in EDL muscles of Akt2\textsuperscript{−/−} mice might be due to a decline in insulin action. Therefore, we examined Akt1 protein levels in tibialis anterior muscles of mice fed a high fat diet for four months. Akt1 protein levels were identical in tibialis anterior muscles from mice fed a high fat diet compared to a normal chow diet (Figure 6), indicating that insulin resistance does not explain the loss of Akt1 protein expression in EDL muscles of Akt2\textsuperscript{−/−} mice. Indeed, this interpretation is not without limitations as our diet-induced insulin resistance study utilized the tibialis anterior muscle rather than the EDL muscle. This limitation is marginalized due to the tibialis anterior and the EDL muscle having almost an identical proportion of type I and II fibers. Another possible limitation is that the insulin resistance of Akt2\textsuperscript{−/−} mice may be distinct from diet-induced insulin resistance as only the latter is associated with obesity.

A particularly interesting and novel finding of the present study is the increase in STARS expression in muscles of Akt2\textsuperscript{−/−} mice. STARS is a muscle specific actin binding protein that activates Rho signaling and stimulates actin polymerization and serum response factor (SRF) dependent gene transcription (2). Recently, STARS expression was associated with insulin resistance, while reducing STARS levels increased Akt signaling and glucose uptake (19). One possibility is that increased STARS expression may have played a role in the decline in Akt phosphorylation and or expression that we observed in muscles of Akt2\textsuperscript{−/−} mice. Alternatively, the severe insulin resistance of the Akt2\textsuperscript{−/−} mice, rather than the whole body ablation of Akt2, may have been responsible for the increase in STARS expression. Further research is needed to
uncover how STARS/SRF dependent gene expression regulates Akt expression and/or phosphorylation as well as insulin action.

The present study demonstrates that aged Akt1−/− mice have significantly lower blood glucose values during an insulin-assisted glucose tolerance test than WT mice. Other investigators have implicated Akt1 in the regulation of glucose metabolism (6, 9, 30). Cho et al.'s (9) original description of young Akt1 null mice revealed a trend for improved glucose tolerance, but this did not reach the threshold for statistical significance. By studying mice at the age of 18 months old, we may have accentuated the deleterious effect Akt1 has on glucose metabolism. The mechanism responsible for the higher insulin sensitivity in Akt1−/− is difficult to explain as it would be expected that less total Akt would promote insulin resistance rather than insulin sensitivity. However, unlike Akt2−/−, muscles from Akt1−/− mice do not have lower levels of pSer473 phosphorylation. It appears that the improved insulin action in Akt1−/− mice is related to their resistance to diet-induced obesity and increased metabolic rate (30). In fact, we show that Akt1−/− mice have a 50% reduction in epididymal white adipose tissue mass, a factor that likely explains a good portion of the increased insulin sensitivity. Further research is needed to identify the mechanism responsible for the enhanced metabolism and obesity resistance of Akt1−/− mice.

Akt signaling promotes muscle hypertrophy, in part, by suppressing the expression of atrogenes (3, 14, 26, 27), a process that involves the transcription factor FOXO. Activation of Akt results in the phosphorylation and nuclear exclusion of FOXO denying the transcription factor’s access to its DNA targets and greatly reducing FOXO-dependent gene transcription. One Akt/FOXO-dependent gene is a muscle specific F-box protein designated MAFbx (muscle atrophy F-box) or atrogin-1, a ubiquitin ligase whose expression is associated with increased protein degradation and muscle atrophy (4, 4, 14, 14). The present study demonstrates that atrogin-1 expression is unchanged in quadriceps muscles as well as soleus and EDL muscles of
Akt1\(^{-}\) or Akt2\(^{-}\) mice (Figures 7A and 8). Although the phosphorylation of Akt is significantly reduced in our Akt2\(^{-}\) mice, the phosphorylation of FOXO3a on Thr\(^{32}\), the residue phosphorylated by Akt, is unaltered (Figure 4). This finding suggests that either the remaining residual Akt activity is sufficient for proper FOXO3a control or that there is a compensatory adaptation that either altered total FOXO3a expression or allowed another kinase to phosphorylate FOXO3a. Unlike the negligible effect of Akt gene ablation on FOXO3a phosphorylation and atrogin-1 expression, the expression of Bnip3 and gabarapl, two autophagic-related atrogenes (23, 32), is significantly increased in quadriceps muscles of Akt1\(^{-}\) and Akt2\(^{-}\) mice (Figures 7B and 7C). This observation is surprising, at least for Bnip3, as it has been shown to control autophagy in skeletal muscle in a FOXO3 dependent manner (23, 32). Unlike the increase in Bnip3 and gabarapl, LC3 expression is not altered in muscles from Akt1 and Akt2 knockout mice, a finding that is consistent with observation that FOXO3a phosphorylation is unaltered. Taken together, our data suggest that Akt controls Bnip3 and gabarapl expression independent of FOXO3a but LC3 expression is regulated by FOXO3a independent of Akt. However, additional studies that directly assess autophagy in muscles that have loss and gain of Akt and FOXO3a function are needed.

Recent evidence demonstrates that FOXO3 regulates skeletal muscle mass by controlling Bnip3 and LC3 expression and autophagy (23, 32). In the present study, we demonstrate that EDL muscle mass is significantly lower in both Akt1 and Akt2 knockouts, but soleus muscle mass is significantly lower only in Akt1 knockouts. The more robust effect of Akt2 ablation on EDL muscle mass compared to soleus muscle mass observed in the present study may be related to the EDL muscle being more susceptible to autophagy than the soleus muscle (24). However, this interpretation is limited by two reasons: 1) we assessed autophagic gene expression in quadriceps muscles rather than EDL and soleus muscle so any relationship with muscle mass must be viewed cautiously; and 2) although EDL and soleus muscle mass is
reduced in Akt knockout mice, the muscle mass/body weight index for EDL and soleus muscles is unchanged indicating a general reduction in total body mass rather than muscle atrophy. Perhaps Akt ablation resulted in a compensatory adaptation that preserves muscle mass even though Bnip3 and gabarapl are elevated.

PERSPECTIVES AND SIGNIFICANCE

The results of the present study demonstrate both divergent and redundant roles for Akt isoforms in regulating whole body insulin action and FOXO-dependent atrogene expression. Our data show that Akt1 knockout mice maintain whole body insulin action and Akt2 knockout mice develop severe insulin resistance. The effects of isoform specific Akt ablation on whole body insulin action is mirrored in the ability of insulin to promote Akt phosphorylation on Thr\(^{308}\) and Ser\(^{473}\), two sites whose phosphorylation is necessary for Akt kinase activity (1). Interestingly, the ablation of Akt2 resulted in the loss of Akt1 expression in EDL muscles, but not soleus muscles, indicating a fiber type specific effect that likely contributed to the reduced whole body insulin action observed in Akt2 null mice. Furthermore, STARS expression was higher in muscles from Akt2\(^{-/-}\) mice, but not Akt1\(^{-/-}\) mice, a process that may not be due to Akt2 ablation per se, but rather due to the insulin resistance resulting from Akt2 knockout. Despite the differences in whole body insulin action and Akt phosphorylation, both Akt1\(^{-/-}\) and Akt2\(^{-/-}\) mice exhibited similar levels of FOXO3a phosphorylation and atrogin-1 expression compared to age-matched wildtype mice. However, expression of the autophagic atrogene, Bnip3 and gabarapl, were increased in muscles from both Akt1 and Akt2 null mice, indicating FOXO3a independent redundancy in Akt1 and Akt2 signaling. Taken together, these results suggest a disassociation of insulin action and Akt/FOXO3a signaling to atrogene.
ACKNOWLEDGEMENTS

The authors are grateful to Dr. Morris Birnbaum of the University of Pennsylvania for donating the Akt knockout mice and to Dr. Thurl E. Harris of the University of Virginia and the Sponsored Research Office at Skidmore College for assistance with manuscript preparation.

GRANT SUPPORT

This work was supported by the National Institutes of Health (1R15AG031504-01), the Skidmore College Summer Research Program, and an American Physiological Society Summer Research Fellowship to Erin Merrell.
REFERENCES


FIGURE LEGENDS

Figure 1. The effect of Akt gene ablation on insulin assisted glucose tolerance. Mice were fasted (six hours) and then given an intraperitoneal injection of insulin (18.0 U/Kg body weight) and glucose (2 mg/g body weight) as described in the Methods. Blood was collected from the tail vein at 0, 15, 30, 45, and 60 min following the insulin/glucose injection and assessed for glucose. The number of mice per group is 4-8. *Akt2−/− mice are significantly different from Akt1−/− and WT mice; +Akt1−/− mice are significantly different from WT mice.

Figure 2. The effect of Akt gene ablation on Akt phosphorylation and expression in extensor digitorum longus muscles (EDL). EDL muscles were isolated intact from wildtype (WT) and Akt null (Akt1−/−, Akt2−/−) mice and incubated in vitro as described in the Methods section. Muscle extracts were prepared and Akt was assessed by immunoblotting with a pThr308 Akt, pSer473 Akt, Akt1, and Akt2 antibodies (Panel A). The pThr308 Akt, pSer473 Akt, Akt1, and Akt2 immunoreactive bands were quantified and data was normalized to total amounts of α-tubulin (Panel B, C, D and E). The number of muscles per group is 4-8. *Significantly different from all other groups. *Significantly different from WT mice. #Significantly different from Akt1−/− mice.

Figure 3. The effect of Akt gene ablation on Akt phosphorylation and expression in soleus muscles. Soleus muscles were isolated intact from wildtype (WT) and Akt null (Akt1−/−, Akt2−/−) mice and incubated in vitro as described in the Methods section. Muscle extracts were prepared and Akt was assessed by immunoblotting with a pThr308 Akt, pSer473 Akt, Akt1, and Akt2 antibodies (Panel A). The pThr308 Akt, pSer473 Akt, Akt1, and Akt2 immunoreactive bands were quantified and data was normalized to total amounts of α-tubulin (Panel B, C, D, and E). The number of muscles per group is 4-8. *Significantly different from all other groups. TSignificantly different from Akt1−/− mice by T-test, but 1 x 3 ANOVA P-value = 0.09.
Figure 4. The effect of Akt gene ablation on FOXO3a phosphorylation on Thr$^{32}$ in extensor digitorum longus (EDL) and soleus muscles. EDL muscles (Panel A) and soleus muscles (Panel B) were isolated intact from wildtype (WT) and Akt null (Akt1$^{-/-}$, Akt2$^{-/-}$) mice and incubated in vitro as described in the Methods section. Muscle extracts were prepared and FOXO3a phosphorylation on Thr$^{32}$ was assessed by immunoblotting with a pThr$^{32}$ FOXO3a antibody. The pThr$^{32}$ FOXO3a immunoreactive bands were quantified and data was normalized to total amounts of $\alpha$-tubulin. The number of muscles per group is 4-8.

Figure 5. The effects of Akt gene ablation on Akt1 expression in quadriceps and extensor digitorum longus (EDL) muscles. mRNA purified from quadriceps muscles of wildtype (WT) and Akt null (Akt1$^{-/-}$, Akt2$^{-/-}$) mice was reverse transcribed and subjected to qPCR as described in the Methods section (Panel A). mRNA purified from EDL muscles of young wildtype (YG-WT) and YG-Akt2$^{-/-}$ mice was reverse transcribed and subjected to qPCR as described in the Methods section (Panel B). Muscle extracts were prepared from EDL muscles of YG-WT and YG-Akt2$^{-/-}$ mice, and Akt1 was assessed by immunoblotting with an Akt1 antibody (Panel C). *Significantly different from muscle from all other groups.

Figure 6. The effects of dietary-induced obesity/insulin resistance on Akt1 expression in skeletal muscles. Tibialis anterior muscles were isolated from control (CON) and high fat diet fed mice (HFD) as described in the Methods section. Muscle extracts were prepared and Akt1 was assessed by immunoblotting with an Akt1 antibody. The Akt1 immunoreactive bands were quantified and data was normalized to total amounts of $\alpha$-tubulin. The number of muscles per group is 5-8.
Figure 7. The effects of Akt gene ablation on atrogene expression in quadriceps muscles. Total mRNA purified from quadriceps muscle of wildtype (WT) and Akt null (Akt1/−, Akt2/−) mice was reverse transcribed and subjected to qPCR as described in the Methods section. *Muscles from Akt1/− and Akt2/− mice are significantly different from muscles of WT mice.

Figure 8. The effect of Akt gene ablation on atrogin-1 levels in extensor digitorum longus (EDL) and soleus muscles. EDL muscles (Panel A) and soleus muscles (Panel B) were isolated intact from wildtype (WT) and Akt null (Akt1/−, Akt2/−) mice and incubated in vitro as described in the Methods section. Muscle extracts were prepared and atrogin-1 expression was assessed by immunoblotting with an atrogin-1 antibody. The atrogin-1 immunoreactive bands were quantified and data was normalized to total amounts of α-tubulin. The number of muscles per group is 4-8.

Figure 9. The effects of Akt gene ablation on STARS expression in quadriceps muscles. Total mRNA purified from quadriceps muscle of wildtype (WT) and Akt null (Akt1/−, Akt2/−) mice was reverse transcribed and subjected to qPCR as described in the Methods section. *Muscles from Akt2/− mice are significantly different from muscles of WT and Akt1/− mice.
Table 1. The physical characteristics of wildtype and AKT knockout mice.

<table>
<thead>
<tr>
<th></th>
<th>Wildtype (n = 21)</th>
<th>AKT1⁻/⁻ (n =12)</th>
<th>AKT2⁻/⁻ (n =4)</th>
<th>1 x 3 ANOVA P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (mg)</td>
<td>37.1 ± 1.5*</td>
<td>25.1 ± 0.6*</td>
<td>30.2 ± 1.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>Soleus Muscle Mass (mg)</td>
<td>18.2 ± 1.0</td>
<td>13.3 ± 1.0#</td>
<td>16.3 ± 2.0</td>
<td>0.0223</td>
</tr>
<tr>
<td>Soleus Mass (mg)/Body Weight (g) Index</td>
<td>0.493 ± 0.041</td>
<td>0.538 ± 0.037</td>
<td>0.416 ± 0.143</td>
<td>0.416</td>
</tr>
<tr>
<td>EDL Muscle Mass (mg)</td>
<td>16.7 ± 1.0*</td>
<td>12.6 ± 1.0</td>
<td>13.0 ± 2.0</td>
<td>0.008</td>
</tr>
<tr>
<td>EDL Mass (mg)/ Body Weight (g) Index</td>
<td>0.491 ± 0.046</td>
<td>0.508 ± 0.030</td>
<td>0.430 ± 0.068</td>
<td>0.576</td>
</tr>
<tr>
<td>Epididymal Adipose Tissue Mass (g)</td>
<td>1.44 ± 0.14*</td>
<td>0.762 ± 0.088</td>
<td>0.333± 0.166</td>
<td>0.0004</td>
</tr>
<tr>
<td>Epididymal Adipose Tissue Mass (g)/ Body Weight Index</td>
<td>50.90 ± 6.54⁺</td>
<td>25.99 ± 3.11</td>
<td>12.65 ± 7.51</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*Significantly different from all groups by Fisher’s LSD, P ≤ 0.05
⁺Significantly different from AKT2⁻/⁻ by Fisher’s LSD, P ≤ 0.05
#Significantly different from WT by Fisher's LSD, P ≤ 0.05
Figure 1

A

Blood Glucose (mg/dL)

Time (min)

WT
AKT1-/-
AKT2-/-

B

Incremental AUC

WT
AKT1-/-
AKT2-/-
Figure 2

A

EDL Muscle

pThr\textsuperscript{308} Blot

pSer\textsuperscript{473} Blot

AKT1 Blot

AKT2 Blot

\(\alpha\)-Tubulin Blot

WT AKT\textsuperscript{1/-} AKT\textsuperscript{2/-}

B

\[ \begin{align*}
\text{pThr}\textsuperscript{308} AKT (\% WT) & \quad 0 & 50 & 100 & 150 & 200 \\
\text{WT} & \bar{\text{AG-WT}} \end{align*} \]

\[ \begin{align*}
\text{AKT1\textsuperscript{1/-}} & \quad \# \\
\text{AKT2\textsuperscript{1/-}} & \quad \bar{\text{AG-WT}} \end{align*} \]

C

\[ \begin{align*}
\text{pSer}\textsuperscript{473} AKT (\% WT) & \quad 0 & 20 & 40 & 60 & 80 & 100 & 120 \\
\text{WT} & \bar{\text{AG-WT}} \end{align*} \]

\[ \begin{align*}
\text{AKT1\textsuperscript{1/-}} & \quad \# \\
\text{AKT2\textsuperscript{1/-}} & \quad \bar{\text{AG-WT}} \end{align*} \]

D

\[ \begin{align*}
\text{AKT1 (\% WT)} & \quad 0 & 20 & 40 & 60 & 80 & 100 & 120 & 140 \\
\text{WT} & \bar{\text{AG-WT}} \end{align*} \]

\[ \begin{align*}
\text{AKT1\textsuperscript{1/-}} & \quad + \\
\text{AKT2\textsuperscript{1/-}} & \quad + \\
\end{align*} \]

E

\[ \begin{align*}
\text{AKT2 (\% WT)} & \quad 0 & 20 & 40 & 60 & 80 & 100 & 120 & 140 \\
\text{WT} & \bar{\text{AG-WT}} \end{align*} \]

\[ \begin{align*}
\text{AKT1\textsuperscript{1/-}} & \quad + \\
\text{AKT2\textsuperscript{1/-}} & \quad \ast \\
\end{align*} \]
Figure 3

A. Soleus Muscle

B. 

C. 

D. 

E. 

Soleus Muscle

pThr\textsuperscript{308} Blot

pSer\textsuperscript{473} Blot

AKT1 Blot

AKT2 Blot

α-Tubulin Blot

WT AKT1\textsuperscript{-/-} AKT2\textsuperscript{-/-}

B. 

C. 

D. 

E. 

WT AKT1\textsuperscript{-/-} AKT2\textsuperscript{-/-}

WT AKT1\textsuperscript{-/-} AKT2\textsuperscript{-/-}

WT AKT1\textsuperscript{-/-} AKT2\textsuperscript{-/-}

WT AKT1\textsuperscript{-/-} AKT2\textsuperscript{-/-}
Figure 4

A  EDL Muscle
pFOXO3a Blot  
α-Tubulin Blot
WT  AKT1⁻⁻  AKT2⁻⁻

B  Soleus Muscle
pFOXO3a Blot  
α-Tubulin Blot
WT  AKT1⁻⁻  AKT2⁻⁻

WT           AKT1⁻⁻  AKT2⁻⁻

pThr³²FOXO3a (%WT)

WT  AKT1⁻⁻  AKT2⁻⁻
Figure 5

A

AKT1 mRNA (%WT)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>AKT1^{-/-}</th>
<th>AKT2^{-/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>120</td>
<td>40</td>
<td>140</td>
</tr>
</tbody>
</table>

B

AKT1 mRNA (%YG-WT)

<table>
<thead>
<tr>
<th></th>
<th>YG-WT</th>
<th>YG-AKT2^{-/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

C

AKT1 Blot

α-Tubulin Blot

<table>
<thead>
<tr>
<th></th>
<th>YG-WT</th>
<th>YG-AKT2^{-/-}</th>
<th>YG-WT</th>
<th>YG-AKT2^{-/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>80</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

* indicates statistical significance.
Figure 6

AKT1 Blot

α-Tubulin Blot

CON       HFD       CON        HFD

AKT1 (%Control)

CON           HFD

0  20  40  60  80  100  120  140
Figure 7

A

Atrogin-1 mRNA (%WT)

WT  AKT1\(^{-/-}\)  AKT2\(^{-/-}\)

0  20  40  60  80  100  120  140

B

Bnip3 mRNA (%WT)

WT  AKT1\(^{-/-}\)  AKT2\(^{-/-}\)

0  50  100  150  200  250  300  350

C

Gabarapl mRNA (%WT)

WT  AKT1\(^{-/-}\)  AKT2\(^{-/-}\)

0  200  400  600  800  1000  1200

D

LC3 mRNA (%WT)

WT  AKT1\(^{-/-}\)  AKT2\(^{-/-}\)

0  20  40  60  80  100  120  140  160
Figure 8

**Panel A**
- EDL Muscle
- Atrogin-1 Blot: WT, AKT1−/−, AKT2−/−
- α-Tubulin Blot: WT, AKT1−/−, AKT2−/−

**Panel B**
- Soleus Muscle
- Atrogin-1 Blot: WT, AKT1−/−, AKT2−/−
- α-Tubulin Blot: WT, AKT1−/−, AKT2−/−

Graphs showing Atrogin-1 expression levels as a percentage of WT for each genotype.
Figure 9

The bar graph shows the STARS mRNA expression levels (%WT) in different genotypes: WT, AKT1−/−, and AKT2−/−. The expression levels are indicated as follows:

- WT: Stabilized expression levels
- AKT1−/−: Slightly reduced expression levels
- AKT2−/−: Significantly increased expression levels

A small asterisk (*) denotes a statistically significant difference compared to the WT group.