Insulin resistance for glucose uptake and Akt2 phosphorylation in the soleus, but not epitrochlearis, muscles of old vs. adult rats

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Intravenous injection of radiolabeled 2-deoxy-D-glucose (2-DG) during the euglycemic-hyperinsulinemic clamp, ESCRIVA and COLLEAGUES directly assessed glucose uptake by both isolated soleus and epitrochlearis muscle. The results suggest the possibility that an age-related decline in pAkt2-Thr308 of the insulin-stimulated slow-twitch muscle may play a role in the insulin resistance in the soleus of old rats. Skeletal muscle insulin resistance in old age is distinctive compared with other insulin-resistant rodent models that are not selective for greater insulin resistance in the soleus vs. the epitrochlearis. Several studies using the euglycemic-hyperinsulinemic clamp procedure at insulin doses expected to suppress hepatic glucose production have revealed an age-related decrement of ~10–20% for 20–24-mo-old rats compared with 6–10-mo-old rats, implicating age-related insulin resistance in peripheral tissues, i.e., especially in skeletal muscle (13, 24, 51). By injecting radiolabeled 2-deoxy-d-glucose (2-DG) during the clamp, ESCHERICH and COLLEAGUES directly assessed glucose uptake rates by several individual skeletal muscles (24). They found that the insulin-stimulated glucose uptake above basal values for the soleus, a predominantly slow-twitch muscle, was substantially lower for 24- vs. 8-mo-old rats (~30–60% lower, depending on the insulin dose). In striking contrast, there was no evidence for an age-related decline in glucose uptake by the predominantly fast-twitch quadriceps muscles from the same animals. This result with aging differs remarkably from other commonly studied rat models of insulin resistance. For example, the relative decrements in glucose uptake in either the obese Zucker rat (64) or in high-fat diet fed rats (45) were quite similar for the soleus compared with predominantly fast-twitch muscles. In other words, age-related insulin resistance in skeletal muscle appeared to be fundamentally different from the two most widely studied models of insulin resistance in rats. In this study, we aimed to better understand the mechanism for this unusual effect of age on insulin resistance in skeletal muscle, a process that has major implications for health.

Skeletal muscle-specific differences in the magnitude of insulin resistance during a euglycemic-hyperinsulinemic clamp could reflect differences related to blood flow, concentrations of humoral factors (e.g., hormones, adipokines, lipids, etc.), neural recruitment, and/or differences intrinsic to the muscle itself. By studying isolated skeletal muscle in vitro, it is possible to separate the intrinsic characteristics of the muscle from short-lived and direct systemic effects. In this context, it is notable that, consistent with the lack of age-related insulin resistance in fast-twitch muscle in vivo, a number of previous studies using isolated preparations of the predominantly fast-twitch epitrochlearis muscle have indicated either very modest or no age-related insulin resistance for rats at 8–13 vs. 20–31 mo (8, 10, 11, 33). Accordingly, in this study, we evaluated the glucose uptake by both isolated soleus and epitrochlearis muscles from adult (9 mo) and old (25 mo) rats using a range of insulin concentrations.

Insulin-stimulated glucose transport in skeletal muscle is mediated via the insulin-regulated GLUT4 glucose transporter protein (30, 43, 65). Previous research has indicated no age-related decrease in GLUT4 protein abundance in rat skeletal muscle between 9 and 31 mo (8). We determined GLUT4 abundance and also assessed several key insulin signaling steps that could potentially account for insulin resistance in the absence of altered GLUT4 levels. Multiple isoforms of Akt are expressed in skeletal muscle, but only Akt2 appears to be important for insulin-stimulated glucose uptake (4, 14, 32, 48, 57), and aging effects on skeletal muscle Akt2, regardless of fiber type, have not previously been reported. We measured Akt1 and Akt2 abundance and insulin-mediated activation of total Akt, Akt1, and Akt2, as well as the abundance of App1, a protein that can regulate Akt activation (60). Phosphorylation of Akt substrate of 160 kDa (AS160) was recently identified as...
a missing link between insulin’s activation of Akt2 and the increased GLUT4 translocation leading to elevated glucose transport rate (7, 12, 38, 58, 59). Insulin resistance for AS160 phosphorylation has been reported in a number of conditions of skeletal muscle insulin resistance (5, 39). Thus we also determined the abundance and insulin-stimulated phosphorylation of AS160. Finally, although Akt2 is important for insulin-induced glucose transport, Akt-independent mechanisms are also required for the full insulin-effect on glucose uptake. A great deal of evidence points to atypical PKC (aPKC) for this Akt-independent portion of insulin-mediated glucose transport in skeletal muscle (26–28). Because the influence of aging on aPKC is unknown, we also assessed the influence of age on aPKC/λ activity in soleus and epimyscicaris muscles. We hypothesized that muscle from older rats will have insulin resistance for key signaling steps (Akt2 phosphorylation, aPKC/λ activity, and AS160 phosphorylation) that correspond to the magnitude of insulin resistance for glucose uptake (i.e., little or no decrease in the epimyscicaris and a large decrease for the soleus).

METHODS

Materials. Unless otherwise noted, all chemicals were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Hannover Park, IL). Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). Reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Hercules, CA). Anti-Akt (no. 9272), anti-phospho Akt^Thr308 (pAkt^Thr308, no. 9271), anti-phospho Akt^Ser473 (pAkt^Ser473, no. 9271), anti-phospho Akt^Thr308 (pAkt^Thr308, no. 9275), anti-phospho-Ser/Thr Akt (no. 2366), and anti-rabbit IgG horseradish peroxidase (no. 7074) were from Cell Signaling Technology (Danvers, MA). Anti-Akt1 (no. sc-7126) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt2 (no. AF23151) was from R&D Biosystems (Minneapolis, MN). Anti-phospho-AKT^Thr308/Thr32 (no. 9801) and anti-AS160 (no. 07-74) were from Millipore (Billerica, MA). Anti-App1 (no. ab5952) was from Abcam (Cambridge, MA). 2-Deoxy-o-[^3H]glucose ([^3H]2-DG) and [^14C]mannitol were from Perkin Elmer (Boston, MA). Reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Hercules, CA). Anti-Akt2 (no. 2366), anti-phospho Akt^Thr308 (pAkt^Thr308, no. 9275), anti-phospho-Ser/Thr Akt (no. 2366), and anti-rabbit IgG horseradish peroxidase (no. 7074) were from Cell Signaling Technology (Danvers, MA). Anti-Akt1 (no. sc-7126) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt2 (no. AF23151) was from R&D Biosystems (Minneapolis, MN). Anti-phospho-AKT^Thr308/Thr32 (no. 9801) and anti-AS160 (no. 07-74) were from Millipore (Billerica, MA). Anti-App1 (no. ab5952) was from Abcam (Cambridge, MA). 2-Deoxy-o-[^3H]glucose ([^3H]2-DG) and [^14C]mannitol were from Perkin Elmer (Boston, MA).

Animal care. The animal protocol for this study was approved by the University of Michigan Committee on Use and Care of Animals. Male Fisher-344 × Brown-Norway, F1 generation rats were obtained from Eli Lilly (Indianapolis, IN) and housed for 1 mo before experimentation. Animals were individually housed in specific pathogen-free conditions in micro-isolation filter top cages and main cages of all rats on the morning of the experimental day between 0700 and 0800. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) between 1030 and 1330. On loss of pedal reflexes, soleus and epimyscicaris muscles were removed and rapidly rinsed in warm (35°C) Krebs-Henseleit buffer (KHB). Muscles were longitudinally split into strips of similar size for each muscle (two strips for each epimyscicaris, and four strips for each soleus). Muscle strips were subsequently placed in vials containing the appropriate media and shaken and continuously gassed (95% O2/5% CO2) in a heated (35°C) water bath. In the first incubation step, all muscles were incubated in vials containing 2 ml of KHB supplemented with 0.1% bovine serum albumin (BSA), 2 mM sodium pyruvate, 6 mM mannitol, and either 0 nM (basal), 1.2 nM (physiological), or 30 nM (supraphysiological) insulin for 30 min. All muscles were then transferred to a second vial containing 2 ml of KHB/BSA solution, the same insulin concentration as the previous step, 1 mM 2-DG (including a final specific activity of 2.25 mCi/mmol[^3H]-2-DG), and 9 mM mannitol (including a final specific activity of 0.022 mCi/mmol[^14C]mannitol) for 20 min. Following the second incubation step, muscles were rapidly blotted on filter paper moistened with ice-cold KHB, trimmed, freeze-clamped using aluminum tongs cooled in liquid nitrogen, and stored at −80°C for later processing and analysis.

Muscle lysate preparation. Frozen muscles were weighed, transferred to pre chilled glass tissue grinder tubes (Kontes, Vineland, NJ), and homogenized in ice-cold lysis buffer (1 ml/muscle) using a glass pestle attached to a motorized homogenizer (Cafrafo, Warton, ON). The lysis buffer contained Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL; no. 78510) supplemented with 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate (Na3VO4), 1 mM β-glycerophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were transferred to microfuge tubes, rotated for 1 h at 4°C, and then centrifuged (15,000 g) for 15 min (4°C) to remove insoluble material. Protein concentration was measured using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL; no. 23225).

Immunoprecipitation. For evaluation of AS160 phosphorylation (PAS-AS160), 200 μg of protein from each sample was combined with a 1:1,000 tiler of PAS antibody and rotated overnight (4°C). Protein G-agarose beads (Upstate, Lake Placid, NY; no. 16-266) were washed three times with lysis buffer and resuspended in lysis buffer to yield a 50% slurry. After initial antibody incubation, 100 μl of 50% slurry mix of protein G-agarose beads were added to the lysate/antibody mix and rotated 2 h at 4°C. Protein G-agarose beads were isolated by centrifugation (4,000 g at 4°C for 1 min) and washed three times in lysis buffer. Antibodies were eluted from the beads with 45 μl of 2× SDS loading buffer and were boiled for 5 min before SDS-PAGE, as described below. For evaluation of Akt1 and Akt2 phosphorylation, ExactaCruz C beads (Santa Cruz Biotechnology; sc-45040) were prepared by washing three times with phosphate-buff ered saline (PBS) and resuspended in 500 μl of PBS. Goat anti-Akt1 or anti-Akt2 antibody (3 μg/sample) was incubated with ExactaCruz C beads (50 μl/sample) and rotated 3 h at 4°C. The antibody-bead complex was then washed three times with PBS and resuspended to yield a 50% slurry. An aliquot 50 μl of the bead-antibody complex was then added to each sample of 200 μg of muscle lysate in 1 ml of PBS and slowly rotated overnight at 4°C. The immunoprecipitation matrix (bead-antibody-antigen) for each sample was washed three times with PBS, aspirating buffer completely after final wash, and then 50 μl of 2× Laemmli buffer was added. Samples were boiled for 5 min and centrifuged, and supernatants were subjected to 10% SDS-PAGE. Resolved proteins were transferred to PVDF membranes that were subsequently incubated in blocking solution [5% nonfat milk protein and Tris-buffered saline (TBS) plus 0.1% Tween-20 (TBST), pH 7.5] for 1 h at room temperature. Blots were then washed (3 × 5 min) in TBST and subsequently incubated in a 5% BSA TBST solution with anti-pAkt^Thr308 (1:1,000) overnight at 4°C. Blots were subjected to secondary antibody incubation and visualized as described below.

Immunoblotting. An equal amount of protein of each sample was mixed with 6× Laemmli buffer boiled for 5 min and separated using SDS-PAGE (7% resolving gel) before being transferred to nitrocel lulose membranes. Membranes were blocked in 5% milk in TBST for 1 h at room temperature and transferred to 5% BSA-TBST with the appropriate primary antibody overnight at 4°C. Membranes were washed three times for 5 min in TBST and incubated with secondary antibody (1:20,000) for 1 h at room temperature. Blots were washed three times for 5 min in TBST, then washed two times for 5 min in TBS (pH 7.5), and then incubated with West Dura Extended Duration Substrate (Pierce; no. 34075) to visualize protein bands. Immunoreactive proteins were quantified by densitometry (AlphaEase FC;
Alpha Innotech, San Leandro, CA). Values are expressed relative to the normalized average of the 30 nM insulin samples on each blot.

Typical PKCζ/free PKCζ activity. Briefly, aPKCζ/free PKCζ were immunoprecipitated from lysates (500 μg) with a rabbit polyclonal antibody (Santa Cruz Biotechnologies; sc-216) that recognizes COOH terminus of PKCζ/free PKCζ. Sepharose-AG beads (Santa Cruz Biotechnologies) were added and incubated for 8 min at 30°C in 100 μl of buffer containing 50 mM Tris.HCl (pH 7.5), 100 μM Na4VO4, 100 μM Na2P2O7, 1 mM NaF, 100 μM PMSF, 4 μg of phosphatidylserine, 50 μM [γ-32P]ATP (NEN Life Science Products, Waltham, MA), 5 mM MgCl2, and, as substrate, 40 μM serine analog of the PKCζ/free PKCζ (BioSource, Carlsbad, CA). After incubation, 32P-labeled substrate was trapped on P-81 filter papers, which were placed in scintillation vials with scintillation cocktail and quantified by liquid scintillation counting.

2-Deoxy-D-glucose uptake. Aliquots (200 μl) of the supernatants were combined in a vial with 10 ml of scintillation cocktail (Research Products International, Mount Prospect, IL) and a scintillation counter (Perkin Elmer, Waltman, MA), and a scintillation counter (Perkin Elmer, Waltman, MA) was used to determine 3H and 14C disintegrations per minute. These values were used to determine [3H]-2-DG uptake as previously described (8, 34). Additionally, to assess insulin’s effects above baseline, Δ values for 2-DG uptake were determined by subtracting the 0 nM (basal) insulin value from the corresponding insulin-stimulated values (1.2 or 30 nM) from the same rat.

Statistical analysis. Two-way ANOVA was used to determine significant main effects (age and insulin concentration) and interactions. Bonferroni t-tests were used for post hoc analysis to identify the source of significant variance (SigmaStat; SPSS, Chicago, IL). For masses (body, epididymal fat pad, or muscle strip) and total abundances, Bonferroni’s test was used to compare adult and old groups. Data are presented as means ± SE. A P value of ≤0.05 was considered statistically significant.

RESULTS

Body mass, epididymal fat pad mass, and muscle strip mass. Body mass was 27% greater (P < 0.05) in old (559.4 ± 7.5 g) vs. adult (441.0 ± 8.5 g) rats. Epididymal fat pad mass was 63% greater (P < 0.05) in old (13.0 ± 0.6 g) vs. adult (8.0 ± 0.4 g) rats. The epididymal fat pad-to-body mass ratio was 28% greater (P < 0.05) in old (0.023 ± 0.001 g/g) vs. adult (0.018 ± 0.001 g/g) rats. The muscle strip mass of the epitrochlearis was 13% greater (P < 0.05) in old (78.6 ± 0.2 mg) vs. adult (69.5 ± 0.2 mg) rats. The mass of soleus strips was not significantly different between old (47.0 ± 0.2 mg) and adult (44.0 ± 0.2 mg) rats.

Total protein abundance. There were no age-related differences in total protein abundance for Appl1, total Akt, Akt1, Akt2, AS160, and GLUT4 in either the epitrochlearis or soleus muscles (Fig. 1).

2-Deoxy-D-glucose uptake. Epitrochlearis 2-DG uptake was not statistically different between adult and old rats with 0 nM insulin (Fig. 2A). Epitrochlearis 2-DG uptake was significantly decreased (P < 0.05) for old vs. adult rats with 1.2 nM (25% decrease) and 30 nM (18% decrease) insulin. To assess insulin’s effects above baseline (Δ-insulin), insulin stimulation of 2-DG uptake above basal values was determined by subtracting the value of the 0 nM group from the values of 1.2 and 30 nM insulin for each rat (Fig. 2B). Δ-Insulin 2-DG uptake values for the epitrochlearis were not significantly different between the adult and old groups with either 1.2 or 30 nM insulin. Soleus 2-DG uptake was significantly decreased (P < 0.05) in old vs. adult rats for each of the three (0 nM, 1.2 nM, and 30 nM) insulin concentrations (decreases of 42, 42, and 35%, respectively; Fig. 2A). There was a trend (P = 0.07) for Δ-insulin 2-DG uptake values in the soleus to be decreased in old vs. adult rats with 1.2 nM (42% decrease) insulin. There was a significant age-related decrease (P < 0.05) for Δ-insulin 2-DG uptake values in the soleus with 30 nM (28% decrease) insulin (Fig. 2B).

Akt phosphorylation. In the epitrochlearis, there was also no age-related difference in phosphorylation of Akt on either the Ser473 residue (pAktSer473; Fig. 3A) or Thr308 residue (pAktThr308; Fig. 3B) with 0 or 1.2 nM insulin, but there was a significant age-related decrease (P < 0.05) in both pAktSer473 and pAktThr308 with 30 nM insulin. Muscle lysates were immunoprecipitated with either Akt1 or Akt2 antibody and immunoblotted with pAktThr308 to determine phosphorylation of Akt1 (pAkt1Thr308, Fig. 4) or Akt2 (pAkt2Thr308, Fig. 5). For the epitrochlearis, there was no age-related difference for pAkt1Thr308 or pAkt2Thr308, regardless of insulin concentration.

In the soleus, there was no age-related difference in pAktSer473 (Fig. 3A) regardless of insulin concentration. There was no age-related difference in pAktThr308 (Fig. 3B) with 0 nM but a significant decrease (P < 0.05) in old vs. adult rats for 1.2 and 30 nM insulin. For the soleus, there was also no age-related difference in pAkt1Thr308 (Fig. 4) or pAkt2Thr308 (Fig. 5) with 0 or 1.2 nM insulin, although it tended (P = 0.109) to be lower in old rats at 1.2 nM. There were significant decreases (P < 0.05) in pAkt1Thr308 and pAkt2Thr308 in the old compared with the adult rats with 30 nM insulin.

AS160 phosphorylation. We evaluated AS160 phosphorylation using three approaches: 1) immunoblotting muscle lysates using the anti-PAS antibody and quantifying the 160-kDa band, which corresponds to the migration of AS160 for rat skeletal muscle (29) (Fig. 6); 2) using the anti-PAS to immunoprecipitate muscle lysate followed by immunoblotting with anti-AS160 (Fig. 7); and 3) using anti-pAS160Thr642 to probe blots of muscle lysates (Fig. 8). None of these analyses revealed any significant age-related differences for soleus or epitrochlearis muscles, regardless of insulin concentration.

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**Fig. 1.** Total protein abundance for Appl1, total Akt, Akt1, Akt2, AS160, and GLUT4. There were no statistically significant differences between the age groups. There was n = 8 muscles per group.

**Fig. 2.** (A) 2-DG uptake was expressed as disintegrations per minute. The 0 nM control (basal) value was subtracted from the 1.2 nM and 30 nM values for 2-DG uptake (A). The 0 nM (basal) value was subtracted from the 1.2 and 30 nM values for 2-DG uptake. There was a trend (P = 0.07) for Δ-insulin 2-DG uptake values in the soleus to be decreased in old vs. adult rats with 1.2 nM (42% decrease) insulin. There was a significant age-related decrease (P < 0.05) for Δ-insulin 2-DG uptake values in the soleus with 30 nM (28% decrease) insulin (B).
There was no age-related difference in \( aPKC/\lambda \) activity in either the epitrochlearis or soleus muscles regardless of insulin concentration (Fig. 9).

**DISCUSSION**

We evaluated the effect of age on glucose uptake, along with several key insulin signaling proteins that regulate glucose uptake, in the predominantly slow-twitch soleus (≈80–93\% type I and 7–20\% type II fibers) (2, 3, 9) and the predominantly fast-twitch epitrochlearis (≈8–15\% type I and ≈85–92\% type II fibers) (49, 63) muscles from adult (9 mo) and old (25 mo) rats. From a functional perspective, the most important new finding in this study was the age-related insulin resistance for glucose uptake by the isolated soleus muscles from 25- vs. 9-mo-old rats. In contrast, for the epitrochlearis, insulin’s ability to increase glucose uptake above basal values was similar between the two age groups, confirming the results from many earlier studies that reported little or no insulin resistance in the epitrochlearis of rats aged 23–31 mo compared with 8–13 mo (8, 10, 11, 33). The present results indicated that age-related insulin resistance for glucose uptake by isolated skeletal muscles is not uniform for all muscles. From a mechanistic perspective, the most important new result was the identification of an age-associated reduction in Akt2 phosphorylation of the insulin-stimulated soleus, but not in the epitrochlearis. Thus the relationship between age and

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**Fig. 2.** A: 2-deoxy-D-glucose (2-DG) uptake in epitrochlearis and soleus muscles with 0, 1.2, or 30 nM insulin. B: the insulin-stimulated increase (\( \Delta \)-insulin) for 2-DG uptake was determined by subtracting the 0 nM insulin value from each muscle in the insulin-stimulated groups (1.2 or 30 nM) within the same rat. Data are means ± SE (n = 10–14 muscles per age and insulin concentration). *P < 0.05 vs. adult in the same insulin treatment group.
Akt2 phosphorylation in each muscle corresponded with the influence of age on insulin-mediated glucose uptake.

The substantial age-related insulin resistance for the soleus without a comparable level of insulin resistance in the epitrochlearis is notable because other commonly studied models of insulin resistance, including the obese Zucker rat or the high-fat diet-fed rat, are not characterized by exaggerated insulin resistance in the soleus compared with other muscles (45, 61, 64). Many previous studies have evaluated glucose uptake for both the soleus and the epitrochlearis from rats with insulin resistance induced by genetic defects, dietary manipulations, exposure to various hormones, or treatment with drugs (17, 18, 25, 35, 41, 42, 47, 52). None of these insulin resistant conditions of diverse origins were characterized by selectively greater insulin resistance for the soleus vs. the epitrochlearis.

Although the selective, age-related insulin resistance in the isolated soleus described in the present study appears to be unusual, the results are reminiscent of those for age effects on in vivo glucose uptake recently reported by Escriva and colleagues (24). They found a substantial, age-related (8 vs. 24 mo) decline in insulin-stimulated glucose uptake by the soleus without any age-related insulin resistance in the predominantly fast-twitch quadriceps muscle in Wistar rats. Thus, whether glucose uptake is measured in vitro or in vivo, substantial age-related insulin resistance was found in the soleus without comparable insulin resistance in predominantly fast-twitch muscle from the same rats. In vivo insulin resistance depends on both the muscle’s intrinsic glucose uptake capacity and the modulation of this capacity by systemic influences, including the circulating concentrations of hormones, adipokines, and lipids, the muscle recruitment pattern, neural input, and the rate of muscle blood flow. The age-related insulin resistance in the isolated soleus reflects the muscle’s intrinsic glucose capacity together with only relatively persistent consequences of the prior in vivo condition.

What intrinsic characteristics of the soleus might account for the age-related insulin resistance? There was no evidence for an age-related decline in GLUT4 protein abundance in either the soleus or the epitrochlearis, consistent with previously published results (8). We also previously reported that GLUT1 protein levels are also unchanged between 9 and 31 mo in epitrochlearis and soleus muscles of Fisher-344 ×
Brown Norway rats (8). In skeletal muscle, insulin increases glucose transport secondary to inducing GLUT4, but not GLUT1, to move from the cell’s interior to the surface membranes (19). The absence of an age-related decrease in GLUT4 protein levels suggests a compromised ability to recruit GLUT4 to the cell surface membranes, perhaps because of defects in insulin signaling or the GLUT4 trafficking machinery. As a first step in assessing possible mechanisms, we focused on studying several key insulin signaling proteins that are important for GLUT4 translocation.

Akt2, but not Akt1 or Akt3, plays a central role in insulin-stimulated glucose transport, and the isoform-specificity of Akt2 for increasing GLUT4 translocation appears to be the consequence of the specific subcellular localization with insulin-stimulation (32). Knockdown of Akt2 by siRNA markedly lowered insulin-stimulated glucose uptake, whereas knockdown of Akt1 had very little effect (37, 40). Akt2-null mice have reduced muscle glucose uptake (14, 48), but Akt1- (15) and Akt3-null (21) mice have normal glucose regulation. Furthermore, the rapid activation of Akt2 is sufficient to increase GLUT4 in cells (50). In the epitrochlearis, there was a small but significant age-related reduction in the total pAktThr308 even though neither pAkt1Thr308 nor pAkt2Thr308 were lower for the old vs. adult group. Skeletal muscle has been reported to express all three Akt isoforms (Akt1, Akt2, and Akt3), and each isoform becomes phosphorylated in response to insulin (6, 16, 57). The observed age-related decrease in total pAktThr308 in the epitrochlearis with 30 nM insulin despite no decrease in pAkt1Thr308 or pAkt2Thr308 in this muscle suggests the possibility that there may have been an age-related decline in pAkt3Thr308. Regardless, alterations in Akt3 activation would not be expected to play an important role in altered insulin sensitivity or glucose metabolism (20, 21). The influence of aging on Akt2Ser473 in the soleus remains to be determined, as does its potential role in the observed insulin resistance for glucose transport. However, phosphorylation of AktThr308, independent of altered phosphorylation on AktSer473, is essential for the full activation of Akt activity and insulin-stimulated glucose transport (1, 44). Based on the available evidence, the reduction in Akt2Thr308 phosphorylation in the soleus of the old compared with adult rats is likely to play a role in the reduced insulin-stimulated glucose uptake.

Fig. 4. pAkt1Thr308 in epitrochlearis and soleus muscles. Muscle lysate was immunoprecipitated with the Akt1 antibody before immunoblotting with the phospho-AktThr308 antibody. Data are means ± SE (n = 10–14 muscles per age and insulin concentration). *P < 0.05 vs. adult in the same insulin treatment group.

Fig. 5. pAkt2Thr308 in epitrochlearis and soleus muscles. Muscle lysate was immunoprecipitated with the Akt2 antibody before immunoblotting with the phospho-AktThr308 antibody. Data are means ± SE (n = 10–14 muscles per age and insulin concentration). *P < 0.05 vs. adult in the same insulin treatment group.

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Akt2 is a Ser/Thr kinase, and AS160 was the first Akt substrate to be identified as a participant in insulin-stimulated GLUT4 translocation and glucose uptake (7, 22, 38, 46, 59). In muscles from Akt2-null mice, insulin-stimulated phosphorylation of AS160 (measured with anti-PAS or anti-pAS160Thr642) was nearly completely eliminated (46). Several conditions with insulin resistance for glucose disposal are characterized by attenuated insulin-stimulated AS160 phosphorylation (5, 39, 62). We expected to find age-related insulin resistance for Akt2 phosphorylation in the soleus to be accompanied by reduced AS160 phosphorylation, but there was no evidence for an age effect on either AS160 abundance or AS160 phosphorylation.

Although AS160 phosphorylation did not track closely with glucose uptake, it is possible that we failed to detect age-related changes in other regulatory aspects of AS160. For example, we measured AS160 phosphorylation with anti-pAS160Thr642 or anti-PAS (which appears to primarily detect pThr642 and possibly pSer588 to a lesser extent) (38). Although Thr642 appears to be an especially important phosphosite for AS160’s role in controlling GLUT4 (59), AS160 can be phosphorylated on several other sites by both Akt and other kinases (31, 59). In addition to phosphorylation, AS160 is regulated by binding to 14-3-3 proteins, and in 3T3-L1 cells the ability of AS160 to bind 14-3-3 proteins appears to be critical for regulating GLUT4 redistribution (53). Finally, it is possible that age can influence the subcellular localization of AS160 (53), which is likely to also be important for its ability to regulate GLUT4. Thus it remains possible that there are age effects on AS160 function that are relevant for the age-related insulin resistance.

An alternative scenario is that the insulin resistance for glucose uptake in the soleus of Old rats was the result of an Akt2-dependent, but AS160-independent mechanism. Other Akt substrates that have been implicated as potential regulators of GLUT4 localization include TBC1D1 (54) and myosin Vb (36). Another possibility is that lesser Akt activation in the old soleus results in attenuated suppression of Akt’s substrate GSK-3, which may in turn induce insulin resistance as the result of greater serine-phosphorylation of the insulin receptor and/or insulin receptor substrates (18, 23).

To probe possible mechanisms for the reduced Akt activation, we studied Appl1, a protein that can interact with and apparently regulate Akt. Reducing Appl1 expression using shRNA in 3T3-L1 cells resulted in reduced insulin-stimulated...
Akt phosphorylation, GLUT4 translocation, and glucose uptake (55). However, there was no evidence of an age effect on an abundance of Appl1 in either muscle. It remains possible that the older rats have altered association of Appl1 with Akt.

Finally, although the decrease in Akt2 activation is an attractive potential candidate for the age-related insulin resistance, the present results do not establish causality, and it remains possible that other mechanisms are essential for the insulin resistance in the soleus. A great deal of evidence indicates that aPKC is an insulin-regulated but Akt-independent signaling protein for increasing GLUT4 translocation and glucose uptake (26–28, 56). For example, muscle-specific (aPKC\(\epsilon\)/\(\alpha\)-null mice) have reduced insulin-stimulated glucose uptake by skeletal muscle (28). However, there was no evidence in the present study for a difference between age groups for aPKC\(\epsilon\)/\(\alpha\) activity in either muscle.

In conclusion, the present study demonstrates age-related insulin resistance for glucose uptake in the isolated soleus muscle from 9- vs. 25-mo-old rats without a comparable decrement of insulin’s ability to increase glucose uptake in the epitrochlearis muscle from the same rats. Reduced activation of Akt2 is supported as a potential component of the mechanism for this selective age effect on the soleus. Our working hypothesis is that the diminished activation of Akt2 results in reduced phosphorylation of AS160 at a site other than Thr642 and/or another Akt2 substrate other than AS160. Future studies using selective pharmacological and/or genetic approaches to test the role of Akt2 in age-related insulin resistance is essential. The substantial insulin resistance in the soleus without comparable insulin resistance in other skeletal muscles of old vs. adult rats is strikingly different from other rodent models for insulin resistance. It would be important for future research to determine whether the age-related insulin resistance is unique to the soleus or is a common feature of aging in other predominantly slow-twitch muscles. If other slow-twitch muscles are characterized by insulin resistance similar to the soleus, it would be valuable to determine whether selective age-related insulin resistance is found in the slow-twitch fibers within predominantly fast-twitch muscles. The present results are a compelling example of the heterogeneity that aging can have on different tissues from the same organism.

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![Fig. 8. AS160 phosphorylation at the Thr642 residue in epitrochlearis and soleus muscles. Data are means ± SE (n = 10–14 muscles per age and insulin concentration).](http://jap.physiology.org/)

![Fig. 9. Atypical protein kinase C (aPKC\(\epsilon\)/\(\alpha\)) activity in epitrochlearis and soleus muscles. Data are means ± SE (n = 10–14 muscles per age and insulin concentration).](http://jap.physiology.org/)

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**Fig. 8.** AS160 phosphorylation at the Thr642 residue in epitrochlearis and soleus muscles. Data are means ± SE (n = 10–14 muscles per age and insulin concentration).

**Fig. 9.** Atypical protein kinase C (aPKC\(\epsilon\)/\(\alpha\)) activity in epitrochlearis and soleus muscles. Data are means ± SE (n = 10–14 muscles per age and insulin concentration).
GRANTS

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REFERENCES

24. Kinnick TR, Youngblood EB, O'Keefe MP, Saengsirisuwon V, Teachey MK, Henriksen EJ. Modulation of insulin resistance and