Calorie restriction leads to greater Akt2 activity and glucose uptake by insulin-stimulated skeletal muscle from old rats

Haiyan Wang¹,², Edward B. Arias¹, and Gregory D. Cartee¹,³,⁴

¹Muscle Biology Laboratory, School of Kinesiology, University of Michigan, Ann Arbor, MI
²College of Physical Education and Health, East China Normal University, Shanghai, China
³Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI
⁴Institute of Gerontology, University of Michigan, Ann Arbor, MI

Running title: Calorie restriction and muscle Akt2 in old rats

Correspondence:
Gregory D. Cartee, Ph.D.
University of Michigan, School of Kinesiology, Room 4745F
401 Washtenaw Avenue, Ann Arbor, MI 48109-2214
Phone: (734) 615-3458
Fax: (734) 936-1925
e-mail: gcartee@umich.edu

Email Addresses: Haiyan Wang, wangha@umich.edu; Edward B. Arias, edarias@umich.edu
Skeletal muscle insulin resistance is associated with many common age-related diseases, but moderate calorie restriction (CR) can substantially elevate glucose uptake by insulin-stimulated skeletal muscle from both young and old rats. The current study evaluated the isolated epitrochlearis muscle from ~24.5 months-old rats that were either fed ad libitum (AL) or subjected to CR (consuming ~65% of ad libitum, AL, intake beginning at ~22.5 months-old). Some muscles were also incubated with MK-2206, a potent and selective Akt inhibitor. The most important results were that in isolated muscles, CR versus AL resulted in: 1) greater insulin-stimulated glucose uptake; 2) that was accompanied by significantly increased insulin-mediated activation of Akt2 as indicated by greater phosphorylation on both Thr\textsuperscript{309} and Ser\textsuperscript{474} along with greater Akt2 activity; 3) concomitant with enhanced phosphorylation of several Akt substrates, including Akt Substrate of 160 kDa on Thr\textsuperscript{642} and Ser\textsuperscript{588}, filamin C on Ser\textsuperscript{2213} and proline-rich Akt substrate of 40 kDa on Thr\textsuperscript{246}, but not TBC1D1 on Thr\textsuperscript{506}; and 4) each of the CR-effects was eliminated by MK-2206. These data provide compelling new evidence linking greater Akt2 activation to the CR-induced elevation of insulin-stimulated glucose uptake by muscle from old animals.

Key Words: glucose transport; insulin signaling; insulin resistance; aging
**INTRODUCTION**

Insulin resistance is associated with many prevalent age-related diseases, including type 2 diabetes, hypertension, cardiovascular disease, some cancers and Alzheimer’s disease (23, 31, 66). Because skeletal muscle is responsible for the largest portion of glucose clearance in response to insulin (21), and skeletal muscle from old individuals remains responsive to improved insulin sensitivity with various interventions (11, 13, 29, 58, 69), advancing the understanding of these interventions may offer useful insights for improving health at older ages. Among these interventions, calorie restriction (CR: ∼20–40% below ad libitum, AL, intake) has been reported to substantially increase whole body insulin sensitivity in multiple species, including humans, non-human primates, dogs, mice and rats (3, 28, 37, 40, 51, 57, 64). CR can also elevate glucose uptake by insulin-stimulated muscle from both young and old rats (13, 54, 55, 58, 61).

Insulin leads to phosphorylation on the two key regulatory sites of the two highly expressed Akt isoforms in skeletal muscle (Akt1 on Thr\textsuperscript{308} and Ser\textsuperscript{473} and Akt2 on Thr\textsuperscript{309} and Ser\textsuperscript{474}). Akt2 is more important than Akt1 for insulin-stimulated glucose transport (6, 8, 27, 36, 41, 46). Earlier research in old rats has demonstrated that CR leads to increased insulin-stimulated Akt phosphorylation measured using methods that do not distinguish the Akt isoform (52, 58, 61). Although previous studies in young mice and rats has implicated Akt2 as a crucial insulin signaling protein for the CR-induced improvement in insulin-stimulated glucose uptake by muscle (41, 55), the influence of CR on Akt2 in old animals has been evaluated for the phosphorylation on Ser\textsuperscript{474} (61), but not on Thr\textsuperscript{309}. This gap in knowledge is important because phosphorylation on both of these sites is required for Akt’s full activation (59). Furthermore, Akt2 activity in muscle has not been reported in response to CR, regardless of age.

Among Akt’s many protein substrates, the Rab GTPase-activating protein known as Akt Substrate of 160 kDa (also called AS160 or TBC1D4) is most convincingly linked to increased
insulin-stimulated glucose transport (9, 12, 14). Akt phosphorylates AS160 on multiple sites, but Thr^{642} and Ser^{588} are the two sites known to be crucial for insulin-stimulated glucose transport (50). TBC1D1, a paralog of AS160 expressed by skeletal muscle that is phosphorylated by Akt on Thr^{596}, can also influence glucose transport (9). The cytoskeletal actin-binding protein known as filamin C (FLNc) is another Akt substrate that is expressed by skeletal muscle (24, 44). FLNc’s known physical interaction with actin is notable given that insulin’s regulation of cytoskeletal actin dynamics is believed to influence both insulin signaling and glucose transport (70). A fourth Akt substrate that is highly expressed by skeletal muscle is proline-rich Akt substrate of 40 kDa (PRAS40) (38). PRAS40 has been most widely studied as a member of mTOR Complex 1 (mTORC1) and mTORC1’s regulation of protein translation and cell growth (62). However, at least under some conditions, PRAS40 abundance and phosphorylation levels appear to be related to insulin-stimulated glucose uptake by skeletal muscle (35, 45).

The current study evaluated the isolated epitrochlearis muscle from ~24.5 months-old rats that were either fed AL or subjected to CR (consuming ~65% of AL intake beginning at ~22.5 months-old). A number of earlier studies have evaluated CR effects on insulin signaling and/or insulin-stimulated glucose uptake by muscle in old rats when CR was initiated at a young age (13, 34, 52, 58, 61, 63). In contrast, much less is known about the effects of CR initiated late in life on insulin signaling and insulin-stimulated glucose uptake. Dean et al. (20) reported enhanced insulin-stimulated glucose uptake with CR initiated in older rats, but insulin signaling measurements were not included in that earlier study. To gain insights into the mechanisms for increased insulin-stimulated glucose uptake when CR is initiated in old age, isolated epitrochlearis muscles were incubated with MK-2206, a potent and selective Akt inhibitor. Muscles were analyzed for: 1) basal and insulin-stimulated glucose uptake; 2) multiple indices of Akt function, including total Akt and Akt2 protein abundance, total Akt Ser^{473} and
Thr\textsuperscript{308} phosphorylation, Akt2 Ser\textsuperscript{474} and Thr\textsuperscript{309} phosphorylation, and Akt2 activity; and 3)
abundance and site-selective phosphorylation of four Akt substrates: AS160 on Ser\textsuperscript{588} and
Thr\textsuperscript{642}, TBC1D1 on Thr\textsuperscript{596}, PRAS40 on Thr\textsuperscript{244} and FLNc on Ser\textsuperscript{2213}.

EXPERIMENTAL PROCEDURES

Materials

All of the chemicals were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hanover Park, IL) unless otherwise noted. The apparatus and reagents for SDS-PAGE and immunoblotting were obtained from Bio-Rad Laboratories (Hercules, CA). Pierce MemCode Reversible Protein Stain Kit (#24585), Pierce BCA Protein Assay and T-PER Tissue Protein Extraction Reagent (#78515) were from Thermo Fisher Scientific (Waltham, MA). Anti-phospho Akt Ser\textsuperscript{473} (pAkt\textsuperscript{Ser473}, #9271), anti-phospho Akt Thr\textsuperscript{308} (pAkt\textsuperscript{Thr308}, #9275), anti-Akt (#4691), anti-Akt2(#3063), anti-phospho AS160 Thr\textsuperscript{642} (pAS160\textsuperscript{Thr642}, #8881), anti-phospho AS160 Ser\textsuperscript{588} (pAS160\textsuperscript{Ser588}, #8730), anti-phospho TBC1D1(pTBC1D1\textsuperscript{Thr596}, #6927), anti-TBC1D1 (#4296), anti-PRAS40 (#2961), anti-phospho PRAS40 Thr\textsuperscript{246} (pPRAS40\textsuperscript{Thr246}, #2997); anti-hexokinase II (#2867) and anti-rabbit IgG horseradish peroxidase conjugate (#7074) were from Cell Signaling Technology (Danvers, MA). Anti-Akt Substrate of 160 kDa (AS160; #ABS54), anti-sheep IgG horseradish peroxidase conjugate (#12-342), PKA inhibitor peptide (#12-151), Akt substrate peptide (#12-340), phosphocellulose paper (#20-134), assay dilution buffer (#20-145) and enhanced chemiluminescence Luminata Forte Western HRP Substrate (#WBLUF0100) were obtained from EMD Millipore (Billerica, MA). Anti-filamin C (FLNc; #sc-48496), anti-goat IgG horseradish peroxidase conjugate (#sc-2020) and anti-mouse IgG horseradish peroxidase conjugate (#sc-2060) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho Filamin-C Ser\textsuperscript{2213} (pFLNc\textsuperscript{Ser2213}, #PB-131) was obtained from Kinasource (Dundee, Scotland, UK). Anti-Akt2 was from R&D Systems (Minneapolis, MN). Akt inhibitor MK-2206
(S1078) was from Selleck Chemicals (Houston, TX). [\( ^{32} \)ATP]-ATP, 2-Deoxy-D-[\( ^{3} \)H]-glucose (\( ^{3} \)H]-2-DG) and \( ^{14} \)C]-mannitol were purchased from Perkin Elmer (Boston, MA). Protein G-magnetic beads (#10004D) were from Life Technologies (Grand Island, NY). Scintillation cocktail (#111195) was from Research Products International (Mount Prospect, IL).

**Animal Treatment**

Animal care procedures were approved by the University of Michigan Committee on Use and Care of Animals. Male Fischer-344 x Brown Norway rats were obtained from the National Institute of Aging (NIA) at approximately 22.5 (22 to 23) months-old. Animals were individually housed in specific pathogen-free conditions in micro-isolation filter top cages, maintained on a 12:12 hour light: dark cycle (lights out at 1700). Rats had ad libitum access to food (NIH 31 chow, Richmond, IN) and water for 1 week acclimation period. During this time-period, baseline food intake was determined each day (food provided minus uneaten food). Following the acclimation period, animals were alternately assigned to the ad libitum (AL) or calorie restriction (CR) group. The AL group had unlimited access to the NIH31 chow throughout the study. The CR group was provided with NIH31/NIA fortified chow (Charles River Laboratories International, Inc.) that was provided by the NIA. The fortified chow contained sufficient vitamin supplementation for the CR rats to ingest vitamins to levels comparable to the AL controls. The CR group was restricted to 65% of AL daily intake for 2 months. Animals were provided their daily food allotment between 1530 and 1630 each day with food intake measured weekly. All rats were weighed once per week. After the 2 month CR period, the AL and CR rats (aged ~25.5 months-old) were euthanized, and the muscle incubation experiment was performed.

**Muscle Dissection and Incubation**

Food was removed from the cages of all rats between 0700 and 0800 on the morning of the muscle experiment. While rats were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg), their epitrochlearis muscles were dissected out. Each muscle
was longitudinally split into two similarly sized strips. These strips were placed in vials including the appropriate media, shaken at 45 revolutions per minute and continuously gassed (95% O₂ - 5% CO₂) in a heated (35°C) water bath. Muscles were incubated in vials containing 2 ml Krebs Henseleit Buffer (KHB) supplemented with bovine serum albumin (BSA; 0.1%), 2 mM sodium pyruvate, 6 mM mannitol, and either dimethyl sulfoxide (vehicle) or MK-2206 (0.5 µM) for 20 minutes. After the first incubation step, muscles were then transferred to second vial containing the identical buffer as the preceding step, with or without a submaximally effective insulin concentration (1.2 nm) for 20 minutes. After the second incubation step, muscles were transferred to a third vial containing 2 ml KHB/BSA, the same concentration of MK-2206 and insulin as previously step, 0.1 mM 2-DG (with final specific activity of 2.25 mCi/mmol [³H]-2-DG), and 9 mM mannitol (with final specific activity of 0.022 mCi/mmol [¹⁴C]-mannitol) for 20 minutes. After the third 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 until subsequent processing and analysis.

**Muscle Lysate Processing**

Frozen muscles were weighed, transferred pre-chilled glass tissue grinding tubes (Kontes, Vineland, NJ), then homogenized in ice-cold lysis buffer (1 ml) using a glass pestle attached to motorized homogenizer (Caframo, Georgian Bluffs, ON). The lysis buffer included TPER supplemented with 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate (NaPP), 1 mM sodium vanadate, 1 mM β-glycerophosphate, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenate were rotated for 1 hour (4°C) prior to centrifugation (15,000 x g for 15 minutes at 4°C). The supernatants were transferred to microfuge tubes and stored at -80°C for subsequent analyses. Protein concentration was measured using the bicinchoninic acid procedure.

**2-Deoxy-D-glucose Uptake**
Aliquots of the supernatants from muscle lysates were pipetted into a vial together with scintillation cocktail. A scintillation counter (Perkin Elmer) was used to determine the $^3$H and $^{14}$C disintegrations per minute. [$^3$H]-2-deoxy-D-glucose (2-DG) uptake was calculated as previously described (10, 30).

**Akt2 Activity**

Akt2 activity was determined according to manufacturer’s instructions (Upstate, Billerica, MA). Briefly, 300 µg protein from each sample was rotated at 4°C for overnight with a rabbit polyclonal antibody that recognized Akt2. Then 50 µl of protein G-magnetic beads were added to the mixture, rotated for 2 hours at 4°C. A magnetic rack (DynaMag-2 Magnet, ThermoFisher) was used for magnetic separation of protein G-immunocomplex. Each immunopellet was washed three times with buffer A (0.5 M sodium chloride, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na$_3$VO$_4$, 0.1% 2-mercaptopoethanol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM NaPP, 10 mM sodium β-glycerophosphate, 0.1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, three times with buffer B (50 mM Tris-HCl, pH 7.5, 0.03% Brij-35, 0.1mM EGTA and 0.1% 2-mercaptoethanol), and twice with assay dilution buffer. Then the immunopellet was combined with Akt substrate peptide, PKA inhibitor peptide and γ-[32P]ATP. After 30 minutes with rotation at 37°C, the reaction was stopped by sequential addition of 20 µl 40% trichloroacetic acid. Next, 40 µl of supernatant was transferred to phosphocellulose paper. After three times washes (twice with 1.5% phosphoric acid and once with acetone), the phosphocellulose paper was placed in the vials containing scintillation cocktail for scintillation counting. Results were expressed relative to the normalized mean of all the samples from each experiment.

**Immunoprecipitation**

The antibodies against pThr$^{308}$ and pSer$^{473}$ on Akt1 also recognize the pThr309 and pSer474 on Akt2, respectively. For evaluation of Akt2 phosphorylation at either Thr$^{309}$ or Ser$^{474}$,
300 µg of protein from each sample was incubated with goat anti-Akt2 antibody, rotated overnight at 4°C. After initial antibody incubation, 50 µl of protein G-magnetic beads were added to the lysate-antibody mixture and rotated for 2 hours at 4°C. The immunoprecipitation matrix (bead-antibody-antigen) for each sample was washed three times with lysis buffer, with complete aspiration of buffer after the final wash, and 40 µl of 2X Laemmli sample buffer was added. Samples were boiled for 5 minutes, centrifuged, subjected to 7% SDS-PAGE and ultimately blotted with anti-pAkt<sup>Thr308</sup> and anti-pAkt<sup>Ser473</sup>.

**Immunoblotting**

An equal amount of protein from muscle lysates was mixed with 6X Laemmli buffer, boiled for 5 minutes, subjected to SDS-PAGE (7% resolving gel), and transferred to polyvinyl difluoride membranes. Equal loading was confirmed using the MemCode protein stain. Membrane were blocked with BSA (5% in TBST, Tris-buffered saline, pH 7.5 plus 0.1% Tween-20) for 1 hour at room temperature, then combined with primary antibody (in TBST plus 5% BSA) overnight at 4°C. TBST was used for membrane washing (3 times for 5 minutes) and then membranes were incubated with the appropriate secondary antibody for 1 hour at room temperature, washed (3 times for 5 minutes) in TBST, followed by washing (3 times for 5 minutes) in TBS (pH 7.5). Enhanced chemiluminescence was used to visualize the protein bands that were quantified by densitometry (AlphaEase FC; Alpha Innotech, San Leandro, CA). Results were expressed relative to the normalized average of all the samples on the blot.

**Statistical Analysis**

Comparisons between two groups was determined by a two-tailed Student’s t-test. One-way analysis variance (ANOVA) was used for muscle comparisons (insulin-stimulated 2-DG uptake and protein analysis by immunoblotting) of more than two groups to identify the source of significant variance. Body mass was statistically analyzed using repeated measures ANOVA. When data were not characterized by normal distribution and/or equal variance, they were
transformed to attain normality and equal variance before performing ANOVA. The Bonferroni
test was used for post-hoc analysis. SigmaPlot version 13.0 (Systat Software, San Jose, CA)
was used for statistical analysis. A P-value < 0.05 was considered statistically significant.

RESULTS

Body Mass

Initial body masses at baseline (week 0) were not significantly different for the AL group
compared to the CR group (Figure 1). Subsequently, the CR rats weighed significantly less
than time-matched AL rats at each time-point during weeks 2 to 8 (P < 0.05). Within the AL
group, body mass was significantly lower (P < 0.05) at baseline (week 0) compared to weeks 2,
3 and 4, but it did not differ significantly between any other weeks during the intervention. The
body mass of CR rats during weeks 1 and 2 differed significantly (P < 0.05) from every other
week of CR, except each other. Also within the CR group, weeks 3 and 5 were significantly
different (P < 0.05) from every week except week 4, and week 4 significantly differed (P < 0.05)
from every other CR week except weeks 3 and 5. Within the CR group, body masses at weeks
6, 7 and 8 were significantly different (P < 0.05) from every other week except each other.

2-Deoxy-D-glucose uptake

There was no significant difference between AL and CR groups for 2-DG uptake in muscles
incubated in the absence of insulin and without MK-2206 (Figure 2). For insulin-stimulated
muscles incubated without MK-2206, the CR group exceeded the AL group (P < 0.05). For
insulin-stimulated CR muscles, incubation with MK-2206 versus without MK-2206 resulted in
significantly decreased 2-DG uptake (P < 0.05). For insulin-stimulated muscles incubated with
MK-2206, there was not a significant diet effect on 2-DG uptake.

Immunoblotting
Equal loading of samples was confirmed based on the MemCode results (2, 16). For all of the phosphorylated proteins, the data were expressed as a ratio of the phosphorylated-to-total protein values.

**Akt**

Total Akt abundance was not significantly different among the groups studied (data not shown). In the absence of both insulin and MK-2206, there was no significant diet effect on either pAkt\textsuperscript{Thr308}/Akt (Figure 3A) or pAkt\textsuperscript{Ser473}/Akt (Figure 3B). For insulin-stimulated muscles incubated without MK-2206, both pAkt\textsuperscript{Thr308}/Akt and pAkt\textsuperscript{Ser473}/Akt ratio were greater for CR versus AL groups (P < 0.001). Incubation of insulin-stimulated CR muscle with MK-2206 significantly reduced both pAkt\textsuperscript{Thr308}/Akt and pAkt\textsuperscript{Ser473}/Akt ratio compared to incubation of insulin-stimulated CR muscle without MK-2206 (P < 0.001). For insulin-stimulated muscles, AL values significantly exceeded CR plus MK-2206 values for both pAkt\textsuperscript{Thr308}/Akt and pAkt\textsuperscript{Ser473}/Akt (P < 0.001).

**Akt2 Abundance and Phosphorylation**

Akt2 abundance did not significantly differ among the groups (data not shown). There was significantly greater pAkt\textsuperscript{Ser474} for CR versus AL groups in the absence of both insulin and MK-2206 (P < 0.01) (Figure 4B). For insulin-stimulated muscles incubated without MK-2206, both pAkt\textsuperscript{Thr309} (P < 0.001; Figure 4A) and pAkt\textsuperscript{Ser474} (P < 0.0001; Figure 4B) were greater for CR versus AL groups. In the insulin-stimulated CR muscles, MK-2206 treatments versus no MK-2206 resulted in significant decreases in both pAkt\textsuperscript{Thr309} (P < 0.01) and pAkt\textsuperscript{Ser474} (P < 0.0001). For insulin-stimulated muscles, pAkt\textsuperscript{Ser474} was significantly lower for CR muscle incubated with MK-2206 versus AL muscles (P < 0.01).

**Akt2 Activity**

Akt2 activity for muscles incubated in the absence of both insulin and MK-2206 was not significantly different between the AL and CR groups (Figure 4C). For insulin-stimulated
muscles incubated without MK-2206, Akt2 activity was greater for the CR versus the AL group (P < 0.01). In the insulin-stimulated CR muscles, incubation with MK-2206 versus without MK-2206 resulted in significantly decreased Akt2 activity (P < 0.001). Akt2 activity was significantly reduced for insulin-stimulated CR muscles incubated with MK-2206 versus insulin-stimulated AL muscles (P < 0.01).

AS160 and TBC1D1

For total AS160 abundance, in the presence of insulin and without MK-2206, the AL values were ~10% greater than CR values (P < 0.05) (data not shown). For both pAS160Ser588/AS160 and pAS160Thr642/AS160, in the absence of both insulin and MK-2206, there were no significant differences between AL and CR groups (Figure 5A and 5B). For insulin-stimulated muscles incubated without MK-2206, pAS160Thr642/AS160 of CR exceeded AL (P < 0.001). For insulin-stimulated CR muscle, pAS160Ser588/AS160 tended to be greater for the CR versus AL group, but this trend was not statistically significant (P = 0.127). In the insulin-stimulated CR muscle, incubation with MK-2206 versus without MK-2206 caused significantly lower pAS160Ser588/AS160 (P < 0.01) and pAS160Thr642/AS160 (P < 0.001). In insulin-stimulated muscles, pAS160Thr642/AS160 of the AL group was greater than the CR plus MK-2206 group (P < 0.001).

No significant differences were detected among the groups studied for either TBC1D1 abundance (data not shown) or pTBC1D1Thr596/TBC1D1 (Figure 5D).

Filamin C

FLNc abundance was not significantly different among the groups studied (data not shown). For FLNcSer2213/FLNc, in the absence of both insulin and MK-2206, there was no significant difference between AL and CR groups (Figure 6A). For insulin-stimulated muscles incubated without MK-2206, pFLNcSer2213/FLNc was greater for CR versus AL (P < 0.01). In insulin-stimulated CR muscles, pFLNcSer2213/FLNc was greater without MK-2206 versus with
MK-2206 (P < 0.05). There was no significant difference in pFLNc\textsuperscript{Ser2213}/FLNc of insulin-stimulated CR muscle incubated with MK-2206 versus insulin-stimulated AL muscles.

PRAS40

For total PRAS40 abundance, there was no significant differences among the groups studied (data not shown). For pPRAS40\textsuperscript{Thr246}/PRAS40 in the absence of both insulin and MK-2206, CR exceeded AL \( (P < 0.05) \) (Figure 7A). For insulin-stimulated muscles incubated without MK-2206, pPRAS40\textsuperscript{Thr246}/PRAS40 was greater for CR versus AL \( (P < 0.001) \). For insulin-stimulated CR muscles, incubation with MK-2206 versus without MK-2206 resulted in significantly lower pPRAS40\textsuperscript{Thr246}/PRAS40 \( (P < 0.001) \). For insulin-stimulated groups, pPRAS40\textsuperscript{Thr246}/PRAS40 from AL muscles exceeded CR muscles incubated with MK-2206 \( (P < 0.001) \).

Hexokinase II

No significant differences were detected for total protein abundance of hexokinase II (data not shown).

DISCUSSION

The most important results of this study were that in the epitrochlearis muscle of 24.5 months-old rats, CR compared to AL resulted in: 1) greater insulin-stimulated glucose uptake; 2) that was accompanied by significantly increased insulin-mediated activation of Akt2 as indicated by greater phosphorylation on both Thr\textsuperscript{309} and Ser\textsuperscript{474} along with greater Akt2 activity; 3) concomitant with enhanced phosphorylation of several Akt substrates, including AS160 on Thr\textsuperscript{642} and Ser\textsuperscript{588}, FLNc on Ser\textsuperscript{2213} and PRAS40 on Thr\textsuperscript{246}, but not TBC1D1 on Thr\textsuperscript{596}; and 4) each of the CR-effects was eliminated by MK-2206, a highly selective Akt-inhibitor. These data provide compelling new evidence linking greater Akt2 activation to the CR-induced elevation of insulin-stimulated glucose uptake by muscle from old animals.
The CR-induced elevation in insulin-stimulated glucose uptake by skeletal muscle was the major functional outcome of the study. In the AL group, the submaximally effective insulin concentration used did not elevate glucose uptake. Previous studies have demonstrated age-related insulin resistance for glucose uptake with submaximally effective insulin concentrations by the isolated epitrochlearis muscle from old AL rats compared to young adult animals (13, 52, 58). It is striking, therefore, that in muscles from old CR rats, insulin-stimulated glucose uptake can be substantially increased to reach values that exceed those found in young AL animals (13). Greater insulin-stimulated glucose uptake for CR versus AL controls has been consistently reported for isolated rat skeletal muscles from old rats (13, 52, 58, 61). CR has also been reported to lead to elevate in vivo glucose uptake by rat skeletal muscle (57, 65). The GLUT4 glucose transporter and hexokinase II are key proteins that regulate skeletal muscle glucose uptake (48). Earlier research has reported no CR-induced increase in skeletal muscle abundance of the GLUT4 glucose transporter in rats, mice or non-human primates (4, 5, 13, 25, 52, 57, 58, 61, 63) or hexokinase II in rats (57, 58, 61). However, CR leads to greater insulin-stimulated translocation of GLUT4 to cell surface membranes of rat skeletal muscle (19). An attractive potential mechanism to explain greater insulin-mediated GLUT4 translocation and glucose transport in the absence of enhanced GLUT4 expression is that CR causes increased insulin signaling in skeletal muscle.

The serine/threonine kinase Akt is a key insulin signaling protein that has been repeatedly reported to be responsive to CR-related effects (41-43, 52, 54-58, 61). Multiple lines of evidence have identified Akt2 as the Akt isoform most important for insulin-stimulated glucose uptake (6, 8, 17, 32, 41, 46). CR leads to increased Akt2 phosphorylation on both of the sites that are required for full insulin-dependent activation in muscles from young rats (42, 54, 55). We recently demonstrated for the first time that CR leads to increased Akt2 Ser\(^{374}\) phosphorylation in insulin-stimulated soleus muscles from old rats (61). However, the current
study was the first to demonstrate in muscles from old rats that CR leads to elevated Akt2 phosphorylation on both Ser\textsuperscript{474} and Thr\textsuperscript{309}. Although Akt or Akt2 phosphorylation is a commonly used surrogate marked for increased enzyme activity, the measurement of Akt2 activity provides a more direct indication of functional capacity. The current data provide the first assessment of muscle Akt2 activity in response to CR, regardless of age. It is important to note that we used recombinant Akt1 and Akt2 protein to confirm that the Akt2 antibody specifically immunoprecipitated Akt2, but not Akt1 (data not shown).

Previous research using Akt2-null mice identified Akt2 as the isoform responsible for AS160 phosphorylation on Ser\textsuperscript{588} and Thr\textsuperscript{642} (39), the two phospho-sites crucial for increased insulin-stimulated glucose uptake (50). The current results, demonstrating greater phosphorylation on both of these AS160 sites in the epitrochlearis, are consistent with results on both sites in the insulin-stimulated epitrochlearis from 9 (54, 55) or 30 months-old CR rats (58). However, these observations differ from the results for Ser\textsuperscript{588} (53) and Thr\textsuperscript{642} (52) in the insulin-stimulated epitrochlearis from 24 months-old CR rats. In each of these experiments, regardless of whether CR-related increases in AS160 phosphorylation were detected (52-55, 58), CR led to increased insulin-stimulated glucose uptake. In addition, an earlier study in obese humans with type 2 diabetes subjected to a low energy diet for 4-35 weeks found elevated insulin-stimulated AS160 Thr\textsuperscript{642} phosphorylation of skeletal muscle along with improved insulin-stimulated glucose disposal (35). To summarize, the results of the current research is consistent with previous research in which CR has usually, but not always, been reported to produce greater AS160 phosphorylation in insulin-stimulated skeletal muscle. Attaining greater AS160 phosphorylation would be expected to favor greater insulin-stimulated glucose uptake, but enhanced phosphorylation of other Akt substrates may also contribute to elevated insulin sensitivity.

Earlier studies reported unaltered TBC1D1 phosphorylation in insulin-stimulated epitrochlearis muscles from either 9 or 24 months-old rats (53, 54). In the current study, CR
also did not lead to greater TBC1D1 phosphorylation on Thr$^{596}$. Previous results demonstrated that TBC1D1 Thr$^{596}$ phosphorylation was substantially reduced in insulin-stimulated muscles from Akt2 null mice (60). However, the enhanced Akt2 activity in the CR group of the current study was insufficient to elevate TBC1D1 Thr$^{596}$ phosphorylation. Experiments using cultured 3T3L1 adipocytes indicated that TBC1D1 can influence insulin-stimulated glucose uptake (49), but mouse skeletal muscle expressing TBC1D1 with a mutation preventing phosphorylation on Thr$^{596}$ did not alter insulin-stimulated glucose uptake (1). The current results do not support the idea that greater TBC1D1 Thr$^{596}$ phosphorylation is essential for the CR-induced increase in insulin sensitivity of rat epitrochlearis muscles.

Consistent with the results of previous research on rats at 9, 24 or 30 months-old (53, 55, 58), in the current study CR led to greater FLNc Ser$^{2213}$ phosphorylation in insulin-stimulated epitrochlearis muscles. FLNc is a muscle-specific actin-binding protein, and the remodeling of actin filaments in insulin-stimulated cells is believed to influence the subcellular distribution of insulin signaling proteins and GLUT4 vesicles (70). In single fibers from rat skeletal muscle, protein abundance of FLNc is highly correlated to GLUT4 abundance (15). As assessed using a cell-free assay, FLNc was phosphorylated to a similar extent by either Akt1 or Akt2 (44), but cell-free assays lack the complexity of intracellular enzyme and substrate interactions. Accordingly, these previous results should be interpreted cautiously. To summarize, although CR has been consistently observed to cause greater insulin-stimulated FLNc phosphorylation in muscles of rats across a wide range of ages, the functional consequences of this outcome remain unknown.

The current study revealed a substantial CR-related increase in PRAS40 Thr$^{246}$ phosphorylation in insulin-stimulated muscles. Earlier research has suggested that insulin-stimulated PRAS40 Thr$^{246}$ phosphorylation, at least under some conditions, appears to correspond with activation of Akt and insulin-stimulated glucose uptake. A high fat diet led to
reduced insulin-stimulated Akt Ser\textsuperscript{473}, PRAS40 Thr\textsuperscript{246} and AS160 Thr\textsuperscript{642} phosphorylation in rat skeletal muscle (45). Insulin-stimulated PRAS40 Thr\textsuperscript{246} phosphorylation in skeletal muscle from obese humans with type 2 diabetes was also increased after 4-35 weeks of reduced energy intake (35). Results from mouse embryonic fibroblasts that were null for Akt1, Akt2 or both Akt1 and Akt2 implicated roles for both isoforms in PRAS40 phosphorylation, with Akt1 being apparently more important (38). In cultured myocytes, PRAS40 knockdown led to decreased insulin-mediated Akt activation and glucose uptake (68), and PRAS40 overexpression resulted in greater insulin-stimulated Akt phosphorylation and glucose uptake (67). Overexpressing PRAS40 that was mutated to prevent Thr\textsuperscript{246} phosphorylation (67) also led to greater insulin-stimulated glucose uptake, indicating that phosphorylation on this site was not essential for this outcome. Because PRAS40 total abundance was unaltered by CR in the current study, it is unclear if the earlier results in cultured myocytes with genetically altered PRAS40 expression are relevant to the CR-induced increase in insulin-stimulated glucose uptake.

Akt’s essential role in the CR-induced enhancement of both protein phosphorylation and glucose uptake in muscles from old rats was revealed for the first time by the MK-2206 data. MK-2206 has been reported to have rather similar binding and inhibitory characteristics for Akt1 compared to Akt2, and to have markedly lower levels of binding to and inhibition of Akt3 compared to both other isoforms (33, 47). MK-2206 eliminated the CR effect on Akt2 phosphorylation and activity, and the significant effects of CR on phosphorylation of AS160, FLNc and PRAS40 were also eliminated concomitant with elimination of CR’s effect on insulin-stimulated glucose uptake. These results are consistent with earlier experiments using young animals in which Akt2 was demonstrated to be essential for CR’s full effect on insulin-stimulated glucose uptake by muscle (41).

The CR-induced increase in Akt activation in insulin-stimulated muscle has been repeatedly observed (18, 41, 42, 54, 55, 58, 61). Insulin receptor substrate-1 (IRS-1)
associated phosphatidylinositol 3-kinase (PI3K) is a key insulin signaling step that is proximal to Akt activation (7). Several earlier studies that assessed adult (5 or 9 month-old) or old (30 month-old) rats found greater Akt activation without enhanced IRS-1-PI3K in insulin-stimulated muscles from CR versus AL animals (18, 54, 58). In contrast, a previous study indicated that IRS-1-PI3K of insulin-stimulated muscles was greater for monkeys (12 years-old) after four years of CR versus AL controls (64). In 3 months-old mice, CR (8 weeks duration) resulted in greater phosphotyrosine-associated PI3K activity compared to AL controls (51). Both insulin-stimulated glucose uptake and Akt phosphorylation were also enhanced in the muscles of the CR compared to AL controls in these earlier studies. Earlier studies have differed with regard to the influence of CR on PI3K, but CR-related increases in both insulin-stimulated Akt activation and glucose uptake have been consistently observed in many studies.

Rats given AL access to food will typically eat sporadically throughout the day with most of their food consumption during the dark cycle (22). Rats consuming a moderate CR diet typically eat most or all of their daily food allotment within ~2-3 hours of being fed (22). Therefore, food intake by CR compared to AL rats is different because of both the amount and the timing of food intake. To assess the effects of CR, independent of temporal differences in food intake, Gazdag et al. (26) evaluated rats under four feeding conditions: AL (unlimited food access), conventional CR (fed once daily at the beginning of the dark cycle), temporally matched AL (fed 3 meals at regular intervals daily) and temporally matched CR (fed 3 meals at regular intervals daily). Insulin-stimulated glucose uptake by muscle was increased in both CR groups compared to both AL groups, and there were no significant differences between either the two CR groups or between the two AL groups. Thus, the CR-related increase in insulin-stimulated glucose uptake was attributable to different amount, not timing, of food intake.

Perspectives and Significance
Moderate CR produces many diverse physiologic outcomes that can positively influence health. One of these outcomes is improved insulin sensitivity that is in large part related to modifications in skeletal muscle. Earlier studies have documented that CR results in enhanced insulin-stimulated activation of the Ser/Thr kinase Akt2 in the skeletal muscle of both young and old animals. The current study provided valuable new evidence that CR initiated during old age can also enhance insulin-stimulated Akt2 activation, and the results support the idea that this CR effect is crucial for enhanced insulin-stimulated glucose uptake in muscle. Although it seems unlikely that a large number of older humans will choose to undergo CR for an extended period of time, elucidating the cellular and molecular mechanisms that underlie important CR-induced health benefits may provide valuable insights that can be used for the development of alternative interventions that can offer similar outcomes.

GRANTS

This research was supported by National Institute on Aging Grant AG-010026.

ACKNOWLEDGEMENTS

We greatly appreciate the valuable technical assistance of Carmen Yu.
FIGURE LEGENDS

Figure 1. Body mass (g) determined at weekly intervals. Data were analyzed using repeated measures ANOVA. Filled circles represent the AL group and open circles represent the CR group. The baseline value (prior to intervention) is represented by week 0. The analysis indicated that *AL exceeds CR for weeks 2 to 8 (P < 0.05); within the AL group, †week 0 was less than weeks 2, 3 and 4 (P < 0.05); within the CR group, ‡week 0 was less than every other week (P < 0.05); within the CR group, §weeks 1 and 2 were different from every week except each other (P < 0.05); within the CR group, ¶weeks 3 and 5 were different from every week except week 4 (P < 0.05); †within the CR group, week 4 was different from every week except weeks 3 and 5; within the CR group, ‡weeks 6, 7 and 8 differed from every week except for each other (P < 0.05). Values are means ± SE; n = 8 per treatment group at each week.

Figure 2. Rates of 2-deoxy-D-glucose (2-DG) uptake in epitrochlearis muscles. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way analysis variance (ANOVA) was used for comparisons of muscles incubated with insulin. The analysis indicated for muscles incubated with insulin revealed that *CR exceeds both AL and CR+MK2206 (P < 0.05). Values are means ± SE; n = 8 per treatment group.

Figure 3. A: Phosphorylated AktThr308/Akt. B: Phosphorylated AktSer473/Akt. C: Representative immunoblots. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way analysis variance (ANOVA) was used for comparisons of muscles incubated with insulin. The analysis indicated for muscles incubated with insulin revealed that *CR exceeds both AL and CR+MK2206 (P < 0.001). In addition for insulin-treated muscles, †CR+MK2206 is less than AL (P < 0.001). Values are means ± SE; n = 8 per treatment.
**Figure 4.** A: Phosphorylated Akt$^{Thr309}$. B: Phosphorylated Akt$^{Ser474}$. C: Akt2 activity. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way analysis variance (ANOVA) was used for comparisons of muscles incubated with insulin. The analysis for muscles incubated without insulin revealed that $\neq$CR exceeds AL for pAkt$^{Ser474}$ (P < 0.01). The analysis for muscles incubated with insulin revealed that *CR exceeds both AL (P < 0.001 for Akt$^{Thr309}$; P < 0.0001 for Akt$^{Ser474}$; P < 0.01 for Akt2 activity) and CR+MK2206 (P < 0.01 for Akt$^{Thr309}$; P < 0.0001 for Akt$^{Ser474}$; P < 0.001 for Akt2 activity). In addition for insulin-treated muscles, †CR with MK+2206 is less than AL (P < 0.01 for Akt$^{Ser474}$; P < 0.01 for Akt2 activity). Values are means ± SE; n = 4-8 per treatment.

**Figure 5.** A: Phosphorylated AS160$^{Ser588}$/AS160. B: Phosphorylated AS160$^{Thr642}$/AS160. C: Representative immunoblots (pAS160$^{Ser588}$, pAS160$^{Thr642}$ and AS160). D: Phosphorylated TBC1D$^{Thr596}$/TBC1D1. E: Representative immunoblots (pTBC1D1$^{Thr596}$ and TBC1D1). Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way analysis variance (ANOVA) was used for comparisons of muscles incubated with insulin. The analysis for muscles incubated with insulin revealed that *CR exceeds CR+MK2206 for pAS160$^{Ser588}$ (P < 0.01) and *CR exceeds both AL and CR+MK2206 for pAS160$^{Thr642}$ (P < 0.001). In addition for insulin-treated muscles, †CR with MK+2206 is less than AL (P < 0.001). Values are means ± SE; n = 8 per treatment.

**Figure 6.** A: Phosphorylated FLNc$^{Ser2213}$/FLNc. B: Representative immunoblots. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way analysis variance (ANOVA) was used for comparisons of muscles incubated with insulin. The analysis
for muscles incubated with insulin revealed that CR exceeds both AL (P < 0.01) and CR+MK2206 (P < 0.05). Values are means ± SE; n = 8 per treatment.

Figure 7. A: Phosphorylated PRAS40Thr642/PRAS40. B: Representative immunoblots. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way analysis variance (ANOVA) was used for comparisons of muscles incubated with insulin. The analysis for muscles incubated without insulin revealed that CR exceeds both AL (P < 0.05). The analysis for muscles incubated with insulin revealed that CR exceeds both AL and CR+MK2206 (P < 0.001). In addition for insulin-treated muscles, CR with MK2206 is less than AL (P < 0.001). Values are means ± SE; n = 8 per treatment.

REFERENCES


69. Xiao Y, Sharma N, Arias EB, Castorena CM, Cartee GD. A persistent increase in insulin-stimulated glucose uptake by both fast-twitch and slow-twitch skeletal muscles after a single exercise session by old rats. *Age (Dordr)* 35: 573-582, 2013.

Figure 1

Body Mass

Time (weeks)

Body Mass (g)

0 1 2 3 4 5 6 7 8

AL
CR
Figure 2

2-DG Uptake

umol x g⁻¹ x 20 min⁻¹

<table>
<thead>
<tr>
<th></th>
<th>AL Basal</th>
<th>AL Insulin</th>
<th>CR Basal</th>
<th>CR Insulin</th>
<th>CR Insulin + MK2206</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference compared to AL Basal.
Figure 3

A) 

B) 

C)
Figure 4

A) pAkt$_{2}^{\text{Thr309}}$

B) pAkt$_{2}^{\text{Ser474}}$

C) Akt2 Activity

[Graphs showing relative units for different conditions: AL (Ad libitum) and CR (Caloric restriction) with Insulin and Insulin + MK2206 effects.]
Figure 5

A) pAS160Ser655/AS160

B) pAS160Thr642/AS160

C) pAS160Ser655

D) pTBC1D1Thr596/TBC1D1

E) pTBC1D1Thr596

TBC1D1

Relative Units

AL  CR

Basal  Insulin  Basal  Insulin  Basal  Insulin +MK2205

Relative Units

AL  CR

Basal  Insulin  Basal  Insulin  Basal  Insulin +MK2205
Figure 6

A) pFLNc_{Ser2213}/FLNc

B) Western Blot for pFLNc_{Ser2213} and FLNc
Figure 7

A) Relative Units

B) pPRAS40\textsuperscript{Thr246} / PRAS40

AL Basal Insulin Basal Insulin Insulin + MK2206

CR