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

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Wang H, Arias EB, Cartee GD. Calorie restriction leads to greater Akt2 activity and glucose uptake by insulin-stimulated skeletal muscle from old rats. Am J Physiol Regul Integr Comp Physiol 310: R449–R458, 2016. First published January 6, 2016; doi:10.1152/ajpregu.00449.2015.—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-mo-old rats that were either fed ad libitum (AL) or subjected to CR (consuming ~65% of ad libitum, AL, intake beginning at ~22.5 mo 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 vs. 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 Thr309 and Ser474 along with greater Akt2 activity, 3) concomitant with enhanced phosphorylation of several Akt substrates, including an Akt substrate of 160 kDa on Thr442 and Ser458, filamin C on Ser2213 and proline-rich Akt substrate of 40 kDa on Thr366, but not TBC1D1 on Thr396; 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.

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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 Ser473 and Thr308 phosphorylation, Akt2 Ser74 and Thr309 phosphorylation, and Akt2 activity; and 3) abundance and site-selective phosphorylation of four Akt substrates: AS160 on Ser588 and Thr442, TBC1D1 on Thr596, PRAS40 on Thr244, and FLNC on Ser2213.

MATERIALS AND METHODS

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 (no. 24585), Pierce BCA assay kit and T-PER (Tissue Protein Extraction Reagent; no. 78515) were from Thermo Fisher Scientific (Waltham, MA). Anti-phospho-Akt Ser473 (pAktSer473; no. 9271), anti-phospho-Akt Thr308 (pAktThr308, no. 9275), anti-Akt (no. 4691), anti-Akt2 (no. 3063), anti-phospho-AS160 Thr442 (pAS160Thr442; no. 8881), anti-phospho-AS160 Ser588 (pAS160Ser588; no. 8730), anti-phospho TBC1D1 (pTBC1D1Thr596; no. 6929), anti-TBC1D1 (no. 4296), anti-PRAS40 (no. 2961), anti-phospho-PRAS40 Thr246 (pPRAS40Thr246; no. 2997), anti-hexokinase II (no. 2867), and anti-rabbit IgG horseradish peroxidase conjugate (no. 7074) were from Cell Signaling Technology (Danvers, MA). Anti-Akt substrate of 160 kDa (AS160; no. ABS54), anti-sheep IgG horseradish peroxidase conjugate (no. 12-342), PKA inhibitor peptide (no. 12-151), Akt substrate peptide (no. 12-340), phosphocellulose paper (no. 20-134), assay dilution buffer (no. 20-145), and enhanced chemiluminescence Luminata Forte Western horseradish peroxidase substrate (no. WBLUP0100) were obtained from EMD Millipore (Billerica, MA). Anti-FLAG M2 antibody (FNc; no. sc-49846), anti-goat IgG horseradish peroxidase conjugate (no. sc-2020), and anti-mouse IgG horseradish peroxidase conjugate (no. sc-2060) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho Filamin-C Ser2213 (pFLNCSer2213, no. PB-131) was obtained from Kinasource (Dundee, Scotland, UK). Anti-Akt2 was obtained from R&D Systems (Minneapolis, MN). Akt inhibitor MK-2206 (no. SI708) was obtained from Selleck Chemicals (Houston, TX). [3H]-glucose ([3H]-2-DG), and [14C]-mannitol were purchased from Selleck Chemicals (Houston, TX). [32ATP]-ATP, 2-Deoxy-D-glucose oleate. 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 1H and 14C disintegrations per minute. [3H]-2-deoxy-o-glucose uptake. The procedure.

Animal treatment. Animal care procedures were approved by the University of Michigan Committee on Use and Care of Animals. Male Fischer-344 × Brown Norway rats were obtained from the National Institute of Aging (NIA) at ~22.5 (22 to 23) mo old. Animals were individually housed in specific pathogen-free conditions in microisolator filter top cages, maintained on a 12:12 h light-dark cycle (lights on at 1700). Rats had ad libitum access to food (NIH 31 chow, Richmond, IN) and water for 1 wk 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), which 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 mo. 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-mo CR period, the AL and CR rats (aged ~25.5 mo 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 of Krebs-Henseleit buffer (KHB) supplemented with BSA (0.1%), 2 mM sodium pyruvate, 6 mM mannitol, and either dimethyl sulfoxide (vehicle) or MK-2206 (0.5 μM) for 20 min. After the first incubation step, muscles were then transferred to a second vial containing the identical buffer as the preceding step, with or without a submaximally effective insulin concentration (1.2 μM) for 20 min. After the second incubation step, muscles were transferred to a third vial containing 2 mL KHB/BSA (same concentration as the previous 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 min. 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 prechilled tissue grinding tubes (Kontes, Vineland, NJ), then homogenized in ice-cold lysis buffer (1 mL) using a glass pestle attached to motorized homogenizer (Cafraizo, German Blissfuls, ON, Canada). The lysis buffer included T-PER 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). Homogenates were rotated at 4°C prior to centrifugation (15,000 g for 15 min 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-o-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 1H and 14C disintegrations per minute. [3H]-2-deoxy-o-glucose (2-DG) uptake was calculated as previously described (10, 30).

Akt2 activity. Akt2 activity was determined according to the manufacturer’s instructions (Upstate, Billerica, MA). Briefly, 300 μg of 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 h 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 (10.5 M sodium chloride, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na2VO4, 0.1% 2-mercaptoethanol, 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 leupeptin, three times with buffer B (50 mM Tris-HCl, pH 7.5, 0.03% Brij-35, 0.1 mM 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 γ32P-ATP. After 30 min 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 washing three times (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
using repeated-measures ANOVA. When data were not characterized
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
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 with the CR
group (Fig. 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
with 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 other 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 differ-
ence between AL and CR groups for 2-DG uptake in muscles incubated in the absence of insulin and without MK-2206 (Fig. 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 vs.
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.

Immunoprecipitation. The antibodies against pThr308 and pSer473
on Akt1 also recognize the pThr309 and pSer474 on Akt2, respectively.
For evaluation of Akt2 phosphorylation at either Thr309 or Ser474, 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 h 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 10 µl of 2X Laemmli sample buffer was added. Samples were
boiled for 5 min, centrifuged, subjected to 7% SDS-PAGE, and
ultimately blotted with anti-pAktThr308 and anti-pAktSer473.

Immunoblotting. An equal amount of protein from muscle lysates
was mixed with 6X Laemmli buffer, boiled for 5 min, subjected
to SDS-PAGE (7% resolving gel), and transferred to polyvinyl difluoride
membranes. Equal loading was confirmed using the MemCode protein
stain. Membranes were blocked with BSA (5% in TBST, Tris-
buffered saline, pH 7.5 plus 0.1% Tween-20) for 1 h at room
temperature, then combined with primary antibody (in TBS-Tween
plus 5% BSA) overnight at 4°C. TBST was used for membrane
washing (3 times for 5 min), and then membranes were incubated with
the appropriate secondary antibody for 1 h at room temperature,
and then 3 times for (5 min) in TBST, followed by washing (three
times for 5 min) 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 deter-
mined by a two-tailed Student’s t-test. One-way 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
relative to the normalized mean of all the samples from each ex-
periment.

Fig. 1. Body mass (g) determined at weekly intervals. Data were analyzed
using repeated-measures ANOVA. Solid circles represent the ad libutum (AL)
group, and open circles represent the calorie restriction (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 (&P < 0.05). Within the CR group,
weeks 6–8 differed from every week except for each other (#P < 0.05). Values
are expressed as means ± SE; n = 8 per treatment group at each week.

Fig. 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 ANOVA was used for comparisons of muscles incubated
with insulin. The analysis revealed that for muscles incubated with insulin
that CR exceeds both AL and CR+MK2206 (*P < 0.05). Values are expressed as
means ± SE; n = 8 per treatment group.

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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 (Fig. 3A) or pAkt\textsuperscript{Ser473}/Akt (Fig. 3B). For insulin-stimulated muscles incubated without MK-2206, both pAkt\textsuperscript{Thr308}/Akt and pAkt\textsuperscript{Ser473}/Akt ratio were greater for CR vs. 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 with 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{Ser473} for CR vs. AL groups in the absence of both insulin and MK-2206 (P < 0.01) (Fig. 4B). For insulin-stimulated muscles incubated without MK-2206, both pAkt\textsuperscript{2Thr309} (P < 0.001; Fig. 4A) and pAkt\textsuperscript{2Ser474} (P < 0.0001; Fig. 4B) were greater for CR vs. AL groups. In the insulin-stimulated CR muscles, MK-2206 treatments vs. no MK-226 resulted in significant decreases in both pAkt\textsuperscript{2Thr309} (P < 0.01) and pAkt\textsuperscript{2Ser474} (P < 0.0001). For insulin-stimulated muscles, pAkt\textsuperscript{2Ser474} was significantly lower for CR muscle incubated with MK-2206 vs. 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 (Fig. 4C). For insulin-stimulated muscles incubated without MK-2206, Akt2 activity was greater for the CR vs. the AL group (P < 0.01). In the insulin-stimulated CR muscles, incubation with MK-2206 vs. without MK-2206 resulted in significantly decreased Akt2 activity (P < 0.001). Akt2 activity was significant reduced for insulin-stimulated CR muscles incubated with MK-2206 vs. 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 pAS160\textsuperscript{Ser588}/AS160 and pAS160\textsuperscript{Thr642}/AS160, in the absence of both insulin and MK-2206, there were no significant differences between AL and CR groups (Fig. 5, A and B). For insulin-stimulated muscles incubated without MK-2206, pAS160\textsuperscript{Thr642}/AS160 of CR exceeded AL (P < 0.001). For insulin-stimulated CR muscle, pAS160\textsuperscript{Ser588}/AS160 tended to be greater for the CR vs. AL group, but this trend was not statistically significant (P = 0.127). In the insulin-stimulated CR muscle, incubation with MK-2206 vs. without MK-2206 caused significantly lower pAS160\textsuperscript{Ser588}/AS160 (P < 0.01) and pAS160\textsuperscript{Thr642}/AS160 (P < 0.001). In insulin-stimulated muscles, pAS160\textsuperscript{Thr642}/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 pTBC1D1\textsuperscript{Thr596}/TBC1D1 (Fig. 5D).

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

![Fig. 3. A: phosphorylated Akt\textsuperscript{Thr308}/Akt. B: phosphorylated Akt\textsuperscript{Ser473}/Akt. C: representative immunoblots. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way 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 expressed as means ± SE; n = 8 per treatment.](http://ajpregu.physiology.org/)
and MK-2206, CR exceeded AL (P < 0.05) (Fig. 7A). For insulin-stimulated muscles incubated without MK-2206, pPRAS40 Thr246/PRAS40 was greater for CR vs. AL (P < 0.001). For insulin-stimulated CR muscles, incubation with MK-2206 vs. without MK-2206 resulted in significantly lower pPRAS40Thr246/PRAS40 (P < 0.001). For insulin-stimulated groups, pPRAS40Thr246/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-mo-old rats, CR compared with AL resulted in 1) greater insulin-stimulated glucose uptake that was accompanied by significantly increased insulin-mediated activation of Akt2 as indicated by greater phosphorylation on both Thr309 and Ser474, along with greater Akt2 activity, 2) concomitant with enhanced phosphorylation of several Akt substrates, including AS160 on Thr642 and Ser588, FLNc on

![Diagram](image-url)

**Fig. 4.** A: phosphorylated Akt2 Thr309, B: phosphorylated Akt2 Ser474, C: Akt2 activity. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way ANOVA was used for comparisons of muscles incubated with insulin. The analysis for muscles incubated without insulin revealed that CR exceeds AL for pAkt2Thr309 (P < 0.01). The analysis for muscles incubated with insulin revealed that CR exceeds both AL (P < 0.001 for Akt2Thr309; P < 0.0001 for Akt2Ser474; P < 0.01 for Akt2 activity) and CR + MK2206 (P < 0.01 for Akt2Thr309; P < 0.0001 for Akt2Ser474; P < 0.001 for Akt2 activity). In addition for insulin-treated muscles, CR with +MK2206 is less than AL (∗P < 0.01 for Akt2Ser474; P < 0.01 for Akt2 activity). Values are expressed as means ± SE; n = 4–8 per treatment.

![Diagram](image-url)

**Fig. 5.** A: phosphorylated AS160Ser588/AS160. B: phosphorylated AS160Thr642/AS160. C: representative immunoblots (pAS160Ser588, pAS160Thr642, and AS160). D: Phosphorylated TBC1D1Thr596/TBC1D1. E: Representative immunoblots (pTBC1D1Thr596 and TBC1D1). Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way ANOVA was used for comparisons of muscles incubated with insulin. The analysis for muscles incubated with insulin revealed that CR exceeds CR + MK2206 for pAS160Ser588 (∗P < 0.01), and CR exceeds both AL and CR + MK2206 for pAS160Thr642 (∗P < 0.001). In addition for insulin-treated muscles, CR with MK2206 is less than AL (∗P < 0.001). Values are expressed as means ± SE; n = 8 per treatment.
expressed as means ± SE; n = 8 per treatment.

Ser2213, and PRAS40 on Thr246, but not TBC1D1 on Thr596, A

Fig. 6. A: phosphorylated FLNcSer2213/FLNc. B: representative immunoblots. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way 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 expressed as means ± SE; n = 8 per treatment.

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 with young adult animals (13, 52, 58). Therefore, it is striking 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 vs. 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 elevated 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 had not reported any CR-induced increase in skeletal muscle abundance of the GLUT4 glucose transporter in rats, mice, or nonhuman 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.

Fig. 7. A: phosphorylated PRAS40Thr642/PRAS40. B: representative immunoblots. Data were analyzed using Student’s t-test for muscles incubated without insulin. One-way 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). 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 expressed as means ± SE; n = 8 per treatment.
both sites in the insulin-stimulated epitrochlears from 9- (54, 55) or 30-mo-old CR rats (58). However, these observations differ from the results for Ser388 (53) and Thr542 (52) in the insulin-stimulated epitrochlears from 24-mo-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 wk found elevated insulin-stimulated AS160 Thr542 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 epitrochlears muscles from either 9- or 24-mo-old rats (53, 54). In the current study, CR also did not lead to greater TBC1D1 phosphorylation on Thr596. Previous results demonstrated that TBC1D1 Thr596 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 Thr596 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 Thr596 did not alter insulin-stimulated glucose uptake (1). The current results do not support the idea that greater TBC1D1 Thr596 phosphorylation is essential for the CR-induced increase in insulin sensitivity of rat epitrochlears muscles.

Consistent with the results of previous research on rats at 9, 24, or 30 mo old (53, 55, 58), in the current study, CR led to greater FLNc Ser2213 phosphorylation in insulin-stimulated epitrochlears 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 enzymes 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 Thr246 phosphorylation in insulin-stimulated muscles. Earlier research has suggested that insulin-stimulated PRAS40 Thr246 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 Ser473, PRAS40 Thr246, and AS160 Thr542 phosphorylation in rat skeletal muscle (45). Insulin-stimulated PRAS40 Thr246 phosphorylation in skeletal muscle from obese humans with Type 2 diabetes was also increased after 4–35 wk of reduced energy intake (35). Results from mouse embryonic fibroblasts that were null for Akt1 and/or 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 Thr246 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 whether 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 with Akt2 and to have markedly lower levels of binding to and inhibition of Akt3 compared with 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 phophatidylinositol 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 mo old) or old (30 mo old) rats found greater Akt activation without enhanced IRS-1-PI3K in insulin-stimulated muscles from CR vs. AL animals (18, 54, 58). In contrast, a previous study indicated that IRS-1-PI3K of insulin-stimulated muscles was greater for monkeys (12 yr old) after four years of CR vs. AL controls (64). In 3-mo-old mice, CR (8 wk duration) resulted in greater phosphotyrosine-associated PI3K activity compared with AL controls (51). Both insulin-stimulated glucose uptake and Akt phosphorylation were also enhanced in the muscles of the CR compared with 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 h of being fed (22). Therefore, food intake by CR compared with 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 three meals at regular intervals daily) and temporally matched CR (fed three meals at regular intervals daily). Insulin-stimulated glucose uptake by muscle was increased in both CR groups compared with 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 the different amount, not the timing, of food intake.

**Perspectives and Significance**

Moderate CR produces many diverse physiological outcomes that can positively influence health. One of these outcomes is improved insulin sensitivity, which 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.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: H.W. and E.B.A. performed experiments; H.W. analyzed data; H.W., E.B.A., and G.D.C. interpreted results of experiments; H.W. prepared figures; H.W. and G.D.C. drafted manuscript; H.W., E.B.A., and G.D.C. approved final version of manuscript; G.D.C. conception and design of research.


Willette AA, Bendlin BB, Starks EJ, Birdsell AC, Johnson SC, Christopher BT, Okonkwo OC, La Rue A, Hermann BP, Kosciel NL, Jonaitis EM, Sager MA, Asthana S. Association of insulin resistance with...


