Greater insulin-mediated Akt phosphorylation concomitant with heterogeneous effects on phosphorylation of Akt substrates in soleus of calorie-restricted rats

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[1]*Muscle Biology Laboratory, School of Kinesiology, University of Michigan, Ann Arbor, Michigan;* [2]*Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan; and* [3]*Institute of Gerontology, University of Michigan, Ann Arbor, Michigan*

Sharma N, Sequea DA, Arias EB, Cartee GD. Greater insulin-mediated Akt phosphorylation concomitant with heterogeneous effects on phosphorylation of Akt substrates in soleus of calorie-restricted rats. *Am J Physiol Regul Integr Comp Physiol* 303: R1261–R1267, 2012. First published October 31, 2012; doi:10.1152/ajpregu.00457.2012.—Akt is a serine/threonine kinase that plays a key role in numerous cellular functions including metabolism, growth, protein synthesis, apoptosis, and cell proliferation. The most consistent and robust effect of moderate calorie restriction (CR; ~60% of ad libitum, AL, food consumption) on insulin signaling in rodent muscle has been enhanced insulin-induced phosphorylation of Akt (pAkt). However, there is limited knowledge regarding the mechanism for this enhancement and its consequences in predominantly slow-twitch muscle. Accordingly, in soleus muscle of 9-mo-old rats, we analyzed the effect of CR and insulin on important signaling events that are proximal to Akt activation including: $pIR^{Tyr1162/1163}$, $pIRS1^{Tyr}$, $pIRS1^{Ser312}$, IRS1-associated phosphatidylinositol 3-kinase activity, or $pPTEN^{Ser380}$. In addition, we analyzed the effect of CR and insulin on Akt substrates that have established or putative roles in glucose metabolism, cellular growth, maintenance of muscle structure, or protein synthesis including $pGSK3\alpha^{Ser21}$, $pGSK3\beta^{Ser9}$, $pTSC2^{Ser939}$, $pP70S6K^{Thr412}$, $pAS160^{Thr642}$, and $pFLNc^{Ser2213}$. The current study demonstrated that the CR-induced increase in pAkt in isolated soleus muscles from 9-mo-old rats can occur without concomitant enhancement of several important insulin signaling events that are proximal to Akt activation. These results suggest that the greater pAkt in the soleus muscles from CR rats was attributable to an alternative mechanism. We also observed that the effects of CR were not uniform for phosphorylation of six insulin-regulated Akt substrates in the soleus. The differential response in phosphorylation by Akt substrates likely has important implications for explaining the complex effect of CR diverse cellular functions.

Rab-GAP glucose uptake; dietary restriction; insulin sensitivity; glucose transport; skeletal muscle

AKT (also known as protein kinase B) is a serine/threonine kinase that plays a central role in regulating a number of cellular functions including metabolism, growth, apoptosis, autophagy, angiogenesis, protein synthesis, and cell proliferation (18, 22, 38). Moderate calorie restriction (CR; ~60% of ad libitum, AL, food consumption) has been demonstrated to cause a striking increase in the insulin-stimulated phosphorylation of Akt in the skeletal muscle of various species (21, 37, 40). In rodents, the most consistent and robust result on isolated muscles has been an elevated insulin-induced phosphorylation of Akt attributed to CR (25–27, 34). The precise molecular mechanism for enhancement of CR of insulin-stimulated Akt phosphorylation is unclear, but we did not find diet-induced differences in key upstream elements of the insulin signaling pathway in isolated rat soleus muscles incubated with a physiological insulin dose (33, 34). Because it seemed possible that CR might alter Akt activation via phosphorylation of other signaling proteins that were not evaluated in these earlier studies, our first aim was to determine in isolated soleus muscles from 9-mo-old rats the influence of CR on insulin-stimulated tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), serine phosphorylation of IRS1, and serine phosphorylation of phosphatase and tensin homologue (PTEN).

In addition to elucidating the specific upstream signaling processes that account for CR's enhancement of Akt phosphorylation, it would be important to identify which of Akt's many protein substrates are influenced by CR. We previously demonstrated that CR enhanced insulin-stimulated threonine phosphorylation of Akt substrate of 160 kDa (AS160; also known as TBC1D4) in the predominantly fast-twitch epitrochlearis muscle (34). AS160 is a Rab GTPase activating protein that is a mediator of insulin's activation of glucose transport (4, 5, 31). We also recently reported that CR resulted in elevated serine phosphorylation of Filamin C (FLNc) in the epitrochlearis of 9-mo-old rats (35). FLNc, an actin-binding protein, has been identified as an Akt substrate that becomes phosphorylated in response to insulin (15, 29). The functional consequences of phosphorylation of FLNc is uncertain, but it is interesting that insulin-regulated remodeling of actin filaments has been implicated in the control of both the spatial localization of insulin signaling proteins and translocation of GLUT4 glucose transporter vesicles (43). However, the effect of CR on FLNc phosphorylation in slow-twitch skeletal muscle has not been studied. Accordingly, our second aim was to determine the influence of CR on insulin-stimulated phosphorylation of multiple Akt substrates, including AS160, FLNc, glycogen synthase kinase-α and -β (GSK-α and GSK-β), tuberous sclerosis complex-2 (TSC2), and the 70-kDa S6 protein kinase (P70S6K), in isolated soleus muscles from 9-mo-old rats.

Finally, our group has previously found that CR effects on the isolated soleus muscle (predominantly slow-twitch) are not identical to effects on predominantly fast-twitch skeletal muscles of rats (33, 34). For example, CR increased insulin-stimulated $pAS160^{Thr642}$ in the predominantly fast-twitch epitrochlearis but not in the soleus from 9-mo-old rats (34). Whereas a large number of studies have characterized CR effects on predominantly fast-twitch skeletal muscles of rats (3, 7–10, 16, 17, 26, 27, 34, 35), a great deal less information is available for predominantly slow-twitch muscles of rats (33, 34). Therefore, our third aim was to measure the effects of CR on other important insulin signaling events ($pIR^{Tyr1162/1163}$,

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IRS1-associated PI3K activity, pAS160^{Thr642}) and glucose uptake by isolated soleus muscles from 9-mo-old rats.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, all chemicals were purchased from Fisher Scientific (Hanover Park, IL) or Sigma Chemical (St. Louis, MO). Reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Hercules, CA). Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). Tissue Protein Extraction Reagent (TPER; no. 78510), bicinchoninic acid (BCA) protein assay (no. 23225), and West Dura Extended Duration Substrate (no. 34075) were from Fisher Scientific (Rockford, IL). MILLIPLEX$_{MAP}$ cell signaling buffer and detection Kit (no. 48-602), MILLIPLEX phospho-MAPmates for IRS1^{panTyr} (no. 46-627), Akt^{Thr308} (no. 46-645), and MILLIPLEX MAP Akt/mTOR Phosphoprotein Panel (no. 48-611; Akt^{Ser473}, GSK3α^{Ser21}, GSKβ^{Ser9}, IR$^{Tyr1162/1163}$, IRS1^{Ser312}, P70S6 kinase^{Thr412}, PTEN^{Ser380}, and TSC2^{Ser939}), and anti-sheep IgG horseradish peroxidase conjugate (no. 12-342) were all purchased from EMD Millipore (Billerica, MA). Anti-phospho AS160 Thr^{642} (pAS160^{Thr642}; no. 3028-P1) was from B-Bridge International (Cupertino, CA). Anti-phospho Akt Thr^{308} (pAkt^{Thr308}; no. 9275), anti-phospho Akt Ser^{473} (pAktSer^{473}; no. 9272), and anti-rabbit IgG horseradish peroxidase conjugate (no. 7074) were from Cell Signaling Technology (Danvers, MA). Anti-Akt2 (no. AF23151) was from R&D Biosystems (Minneapolis, MN). Anti-phospho Filamin C^{Ser2213} (pFLNc^{Ser2213}; PB-131) was from Kinasource (Dundee, Scotland, UK). 2-Deoxy-D-[^{3}H]glucose (2-[^{3}H]DG), [^{14}C]mannitol, and γ-[^{32}P]ATP were from Perkin Elmer (Boston, MA). Sepharose-A beads (no. 17-0469-01) were from GE Healthcare (Piscataway, NJ). Phosphatidylinositol was from Avanti Polar Lipids (Alabaster, AL). TLC plates (no. 4865-821) were from Whatman (Piscataway, NJ).

Animal treatment. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. Male Fischer 344 × Brown Norway rats, both CR rats and their AL controls were obtained at 8 mo of age from National Institute of Aging (NIA) Calorie Restricted Rodent Colony. The CR protocol was initiated at 14 wk of age in the CR group by the NIA as previously described (34). Upon arrival in Ann Arbor, rats were housed at the University of Michigan for ~1 mo before experimentation. During this time the rats were housed individually in shoebox cages and maintained on a 12–12 h light-dark cycle (lights out at 17:00 h) in specific pathogen-free conditions and had free access to water. Rats were provided chow (AL: NIH31 chow; CR: NIH31/NIA Fortified chow) and maintained on their respective feeding protocol (AL: free access to chow; CR: ~60–65% of AL consumption).

Muscle dissection and incubation. Rats were euthanized when they were ~9 mo of age. Food was removed from the cages of all rats on the morning of the experimental day between 07:00 and 08:00 h. Soleus muscle dissection was performed as previously described (34), and the resultant soleus strips were placed in vials containing the appropriate media as described below, shaken at 45 rpm, continuously gassed (95% O$_2$-5% CO$_2$), and heated (35°C) in a water bath. In the first incubation step, all muscles were incubated in vials containing 2 ml Krebs-Henseleit buffer (KHB) supplemented with 0.1% bovine serum albumin (BSA), 2 mM sodium pyruvate, 6 mM mannitol as a rinse step for 30 min. In the second incubation step, muscles were incubated in vials containing 2 ml KHB supplemented with 0.1% BSA, 2 mM sodium pyruvate, 6 mM mannitol, and either 0 nM (basal) or 1.2 nM insulin for 30 min. Muscles were either transferred to a third vial containing 2 ml of KHB-BSA solution, the same insulin concentration as the previous step with 0.1% BSA, 1 mM 2-DG, and 9 mM mannitol, or to assay for 2-DG uptake, transferred to a third 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 2-[^{3}H]DG), and 9 mM mannitol (including a final

specific activity of 0.022 mCi/mmol [^{14}C]mannitol) for 20 min. After the third incubation step, the 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 microfuge tubes, and homogenized in ice-cold lysis buffer (1 ml/muscle) using a TissueLyser II (Qiagen, Valencia, CA). The lysis buffer contained tissue protein extraction reagent (TPER) supplemented with 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM β-glycerophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. 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 BCA method.

Immunoprecipitation. Evaluation of Akt2 phosphorylation at either the Thr^{308} or Ser^{473} residue was performed as has been previously described (34).

Immunoblotting. Western blotting procedures were performed as have been previously described (34). Immunoreactive proteins were quantified by densitometry (AlphaEase FC; Alpha Innotech, San Leandro, CA). Values are expressed relative to the normalized average of the basal samples on each blot.

Multiplex analysis. Multiplex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), as described previously (36).

IRS-1-associated PI3K activity. IRS1-phosphatidylinositol 3-kinase (PI3K) activity in the soleus muscle was determined as previously described (10, 34).

2-DG uptake. The calculation of 2-[^{3}H]DG uptake from skeletal muscle lysates was performed as has been previously described (2, 19).

Statistical analysis. Two-way ANOVA was used to determine the main effects of insulin (0 or 1.2 nM) and diet (AL or CR) and interactions, and a Student's *t*-test was used to compare body masses between AL and CR groups (SigmaPlot version 11.0; Systat Software, San Jose, CA). Data are presented as means ± SE. A *P* value ≤0.05 for was considered statistically significant.

RESULTS

Proximal insulin signaling. There was a significant ($P \leq$ 0.05) main effect of insulin (1.2 nM > 0 nM) for pIR$^{Tyr1162/1163}$ (Fig. 1*A*). There was no significant main effect of diet or insulin for pIRS1$^{pan\ Tyr}$ (Fig. 1*B*). There was a significant ($P \leq$ 0.05) main effect of insulin (1.2 nM > 0 nM) for pIRS1^{Ser312} (Fig. 1*C*). There were significant ($P \leq$ 0.05) main effects of diet (CR < AL) and insulin (1.2 nM > 0 nM) for IRS-1 associated PI3K activity (Fig. 1*D*). There was no significant main effect of diet or insulin for pPTEN^{Ser380} (Fig. 1*E*).

Akt phosphorylation. There were significant ($P \leq$ 0.05) main effects of diet (CR > AL) and insulin (1.2 nM > 0 nM) for pAkt^{Thr308} (Fig. 2*A*). There was a significant ($P \leq$ 0.05) main effect of insulin for pAkt^{Ser473} (Fig. 2*B*). There were significant ($P \leq$ 0.05) main effects of diet (CR > AL), insulin (1.2 nM > 0 nM), and an interaction (AL and CR groups are similar with 0 nM insulin, however, CR is much greater than AL with 1.2 mM insulin) for pAkt2^{Thr308} (Fig. 2*C*). There were significant ($P \leq$ 0.05) main effects of diet (CR > AL), insulin (1.2 nM > 0 nM), and an interaction (AL and CR groups are similar with 0 nM insulin, however, CR is much greater than AL with 1.2 mM insulin) for pAkt2^{Ser473} (Fig. 2*D*).

Akt substrate phosphorylation. There were significant ($P \leq$ 0.05) main effects of diet (CR < AL) and insulin (1.2 nM > 0 nM) for pAS160^{Thr642} (Fig. 3*A*). There was a significant ($P \leq$ 0.05) main effect of insulin (1.2 nM > 0 nM) and an
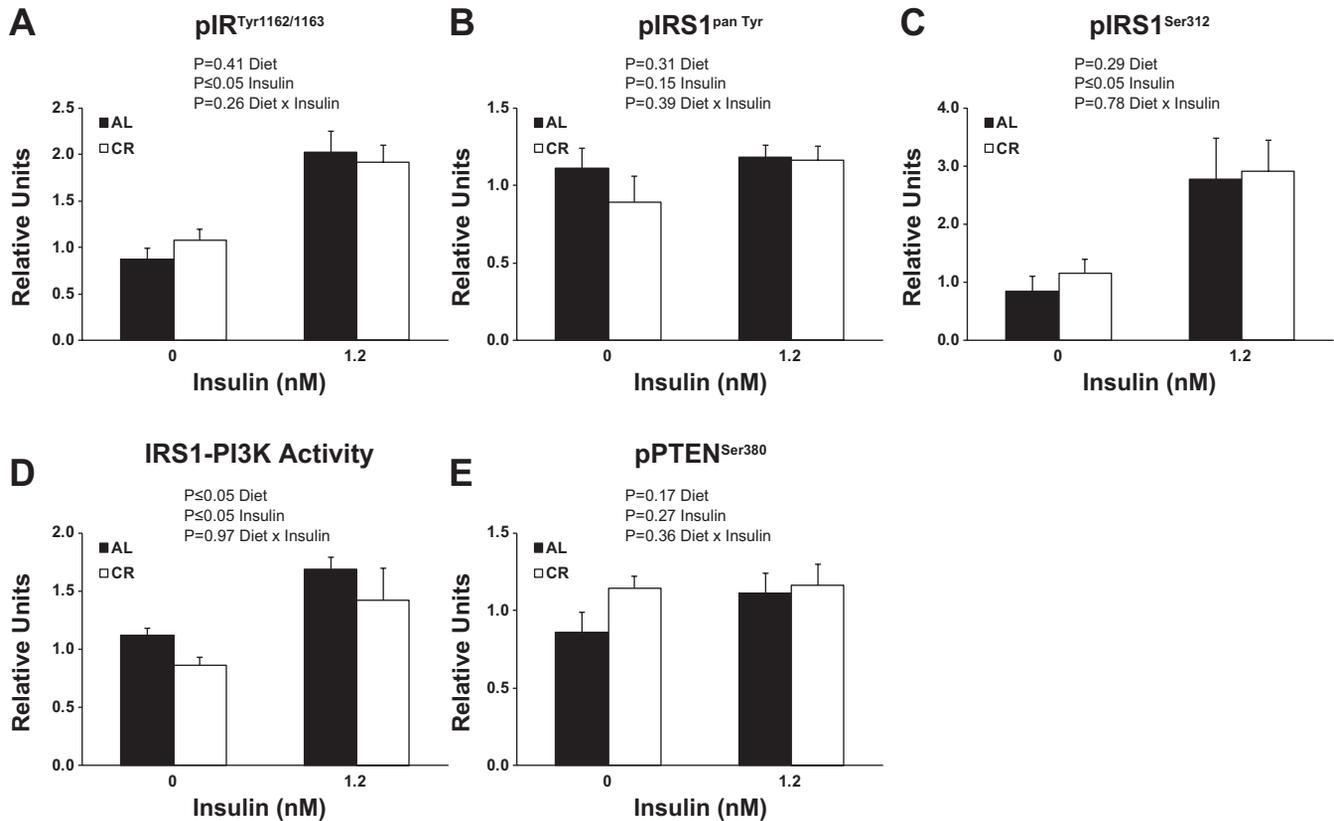
Fig. 1. Phosphorylation of insulin receptor substrate (IR)$^{Tyr1162/1163}$ (A), IRS1$^{pan\ Tyr}$ (B), IRS^{Ser312} (C), IRS1-associated phosphatidylinositol 3-kinase (PI3K) activity (D), and phosphorylation phosphatase and tension homologue (PTEN)^{Ser380} (E). CR, calorie-restricted group; AL, ad libitum food consumption group. Main effects of diet, insulin, and diet $\times$ insulin interactions are reported from two-way ANOVA analysis. $P \leq 0.05$ is considered statistically significant. Values are means $\pm$ SE; $n = 7$–18 per treatment group.

interaction (AL and CR groups are similar with 0 nM insulin, however, CR is much greater than AL with 1.2 mM insulin) for pFLNc^{Ser2213} (Fig. 3B). When Δinsulin values were calculated (insulin stimulated $-$ basal values), there was a significant ($P \leq 0.05$) effect of CR on pFLNc^{Ser2213}. There was a significant ($P \leq 0.05$) main effect of insulin (1.2 nM > 0 nM) for pGSK3α^{Ser21} (Fig. 4A). There was a statistically nonsignificant trend for a main effect of diet ($P = 0.06$) and a significant ($P \leq 0.05$) main effect of insulin (1.2 nM > 0 nM) for pGSK3β^{Ser9} (Fig. 4B). There was a statistically nonsignificant trend for a main effect of diet ($P = 0.06$) and a significant ($P \leq 0.05$) main effect of insulin (1.2 nM > 0 nM) for pTSC2^{Ser939} (Fig. 4C). There were significant ($P \leq 0.05$) main effects of diet (CR > AL) and insulin (1.2 nM > 0 nM) for pP70S6K^{Thr412} (Fig. 4D).

2-DG uptake. There were significant ($P \leq 0.05$) main effects of diet (CR > AL) and insulin (1.2 nM > 0 nM) for 2-DG uptake (Fig. 5).

DISCUSSION

The current study reinforced and extended the results of prior research that has consistently reported that CR enhances insulin-stimulated Akt phosphorylation in isolated rat skeletal muscle (25, 26, 34, 35). Earlier studies indicated that the effects of CR on Akt in isolated rat skeletal muscle were not accompanied by significantly greater activation of key upstream insulin signaling steps (tyrosine phosphorylation of IR

or IRS1, and IRS1-associated PI3K activity) that regulate Akt phosphorylation (7, 8, 34). In the current study we further advanced previous knowledge by also reporting for the first time in isolated rat soleus muscles that CR did not induce a greater activation of IRS1^{Ser312} phosphorylation or PTEN^{Ser380} phosphorylation. These novel results suggest that the mechanism for the CR-induced increase in Akt activation is not dependent on greater activation of these important proximal insulin signaling steps that have been demonstrated to regulate Akt.

The lack of a diet effect on insulin-stimulated IRS1-associated PI3K activity of the soleus is similar to our previous results in a different cohort of rats undergoing identical dietary treatment (34). Other work from our group using predominantly fast-twitch rat muscles has also shown no CR effect on insulin-stimulated PI3K activity (7, 9, 10). However, it has been reported that CR enhanced insulin-stimulated pY-associated PI3K activity in isolated soleus muscles from mice (32), as well as IRS1-associated PI3K activity in vastus lateralis muscles from monkeys under hyperinsulinemic-euglycemic clamp conditions (40). It is unclear if the differing results for CR effects on PI3K are related to differences in species, insulin concentration, or other experimental differences. Nonetheless, our group previously reported that the activity of atypical PKC (which, along with Akt, is a downstream target of PI3K) was not affected by CR, consistent with the idea that CR does not appear to enhance IRS1-PI3K in isolated rat muscle (34).
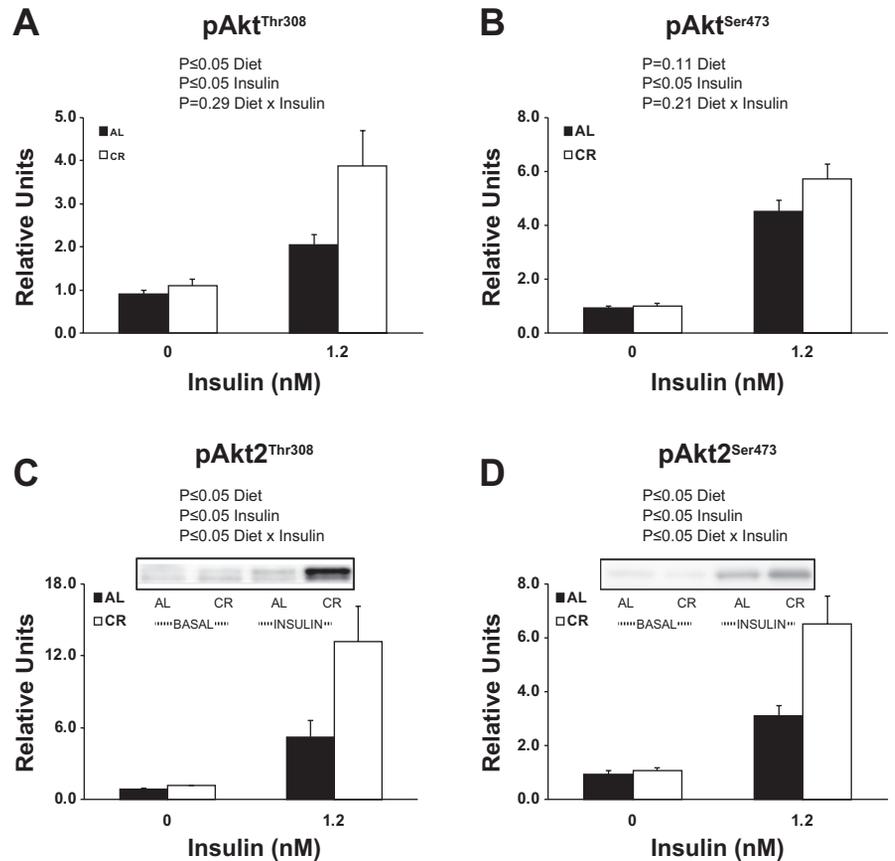
Fig. 2. Phosphorylation of Akt^{Thr308} (A), Akt^{Ser473} (B), $Akt2^{Thr308}$ (C), and $Akt2^{Ser473}$ (D). Main effects of diet, insulin, and diet $\times$ insulin interactions are reported from two-way ANOVA analysis. $P \leq 0.05$ is considered statistically significant. Values are means $\pm$ SE; $n = 7–18$ per treatment group.

PTEN is another protein that could potentially influence Akt phosphorylation. Phosphorylation of PTEN on Ser380 reduces PTEN-mediated dephosphorylation of (3,4,5)-trisphosphate (20, 24, 42), thus favoring greater activation of Akt. PTEN mutations in humans have been demonstrated to enhance insulin sensitivity in skeletal muscle (30). However, in the current study, CR did not alter $pPTEN^{Ser380}$ indicating that its activity is unlikely to account for the CR effects on Akt phosphorylation in the soleus.

The lack of evidence for significant CR effects on proximal insulin signaling steps in isolated rat skeletal muscle begs the question, what might account for the greater Akt that is consistently found in rat skeletal muscle? One possibility is that CR may influence the subcellular localization of Akt. Full activation requires Akt insertion into the plasma membrane (1). CR may increase the amount of Akt at the plasma membrane leading to greater Akt activation. However, it is unclear what might account for such a change in Akt localization with CR. There is evidence that CR may alter the binding of Akt to proteins that lead to greater phosphorylation thus enhancing Akt signaling. In epitrochlearis muscles from 9-mo-old rats, CR versus AL animals had greater heat shock protein (HSP)90-bound to Akt (based on coimmunoprecipitation) concomitant with increased Akt phosphorylation (34). However, there was
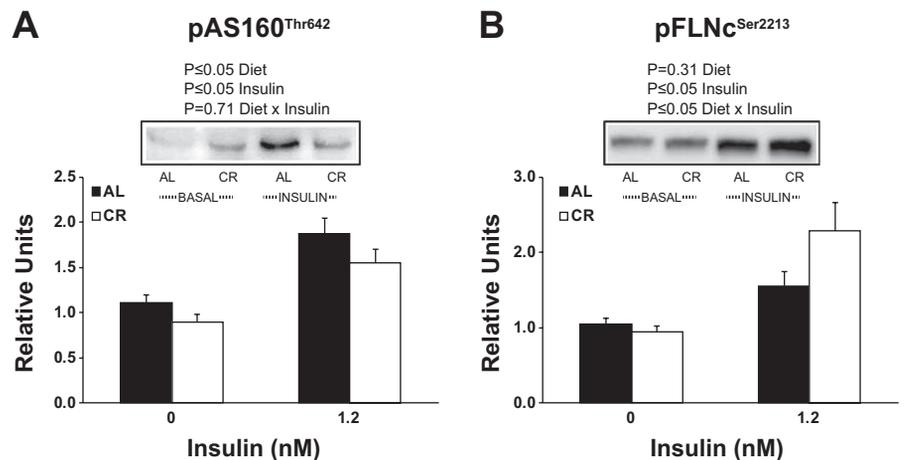

Fig. 3. Phosphorylation of $AS160^{Thr642}$ (A) and Filamin $C^{Ser2213}$ (B). Main effects of diet, insulin, and diet $\times$ insulin interactions are reported from two-way ANOVA analysis. $P \leq 0.05$ is considered statistically significant. Values are means $\pm$ SE; $n = 8–14$ per treatment group.

A pGSK3α^Ser21

P=0.24 Diet
P≤0.05 Insulin
P=0.56 Diet x Insulin

B pGSK3β^Ser9

P=0.06 Diet
P≤0.05 Insulin
P=0.44 Diet x Insulin

C pTSC2^Ser939

P=0.06 Diet
P≤0.05 Insulin
P=0.50 Diet x Insulin

D p70S6K^Thr412

P≤0.05 Diet
P≤0.05 Insulin
P=0.52 Diet x Insulin

Fig. 4. Phosphorylation of GSK3α^{Ser21} (*A*), GSK3β^{Ser9} (*B*), TSC2^{Ser939} (*C*), P70S6K^{Thr412} (*D*). Main effects of diet, insulin, and diet × insulin interactions are reported from two-way ANOVA analysis. $P \leq 0.05$ is considered statistically significant. Values are means ± SE; $n = 8$ per treatment group.

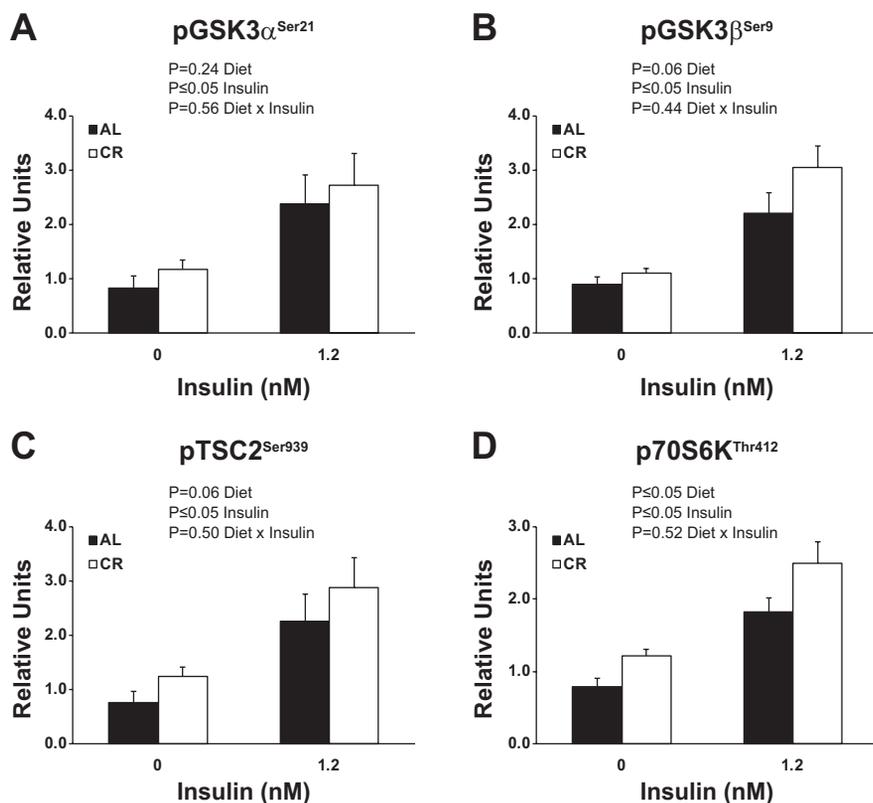no evidence of increased HSP90-Akt association in the soleus of CR rats in this earlier study. It remains possible that CR may be improving Akt signaling in the soleus through alterations in the binding to other protein partners. Because phosphorylation of proteins depends on the balance between the actions of kinases and phosphatases, another possibility is that CR reduces the rate of Akt dephosphorylation. However, Sharma et al. (34) found in soleus and epitrochlearis muscles from 9-mo-old rats that there was no CR-related difference in Akt association with protein phosphatase 2A, a key Ser/Thr protein phosphatase that dephosphorylates Akt (28). Identification of

2-DG Uptake

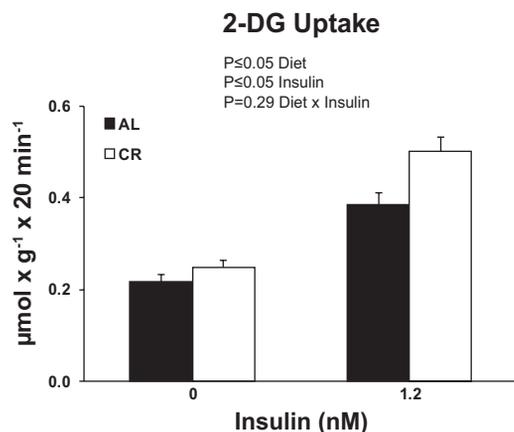P≤0.05 Diet
P≤0.05 Insulin
P=0.29 Diet x Insulin

Fig. 5. Rates of 2-deoxy-D-glucose (2-DG) uptake in soleus muscle strips incubated without (0 nM) or with (1.2 nM) insulin. Main effects of diet, insulin, and diet × insulin interactions are reported from two-way ANOVA analysis. $P \leq 0.05$ is considered statistically significant. Values are means ± SE; $n = 23$–24 per treatment group.

CR-mediated factors that associate with Akt and enhance its actions should be a target of future study.

We measured the phosphorylation status of several insulin-responsive Akt substrates. Phosphorylation of AS160 on its Thr642 site is important for insulin-stimulated GLUT4 translocation and glucose uptake (31). CR did not further enhance insulin-stimulated AS160^{Thr642} phosphorylation, which is consistent with a prior publication in isolated soleus muscles (34). We have previously reported that CR enhanced insulin-stimulated AS160^{Thr642} phosphorylation in isolated epitrochlearis (predominantly fast-twitch) muscles (34, 35). The current findings support the idea that there may be a muscle-specific, and possibly a fiber-type specific, response to the effect of CR on AS160^{Thr642} phosphorylation. The Thr642 phosphosite along with the Ser588 phosphosite on AS160 is crucial for insulin-stimulated glucose uptake (31). We also previously reported that CR does not enhance insulin-stimulated AS160^{Ser588} phosphorylation in isolated rat soleus muscle (34). The increased insulin-stimulated glucose uptake and Akt phosphorylation for the soleus of CR compared with AL rats was also consistent with earlier results for the isolated rat soleus muscle (34). Taken together, it seems likely that CR may be influencing glucose uptake in the soleus as the result actions on phosphorylation of Akt substrates other than AS160.

FLNc is an actin-binding protein that is believed to stabilize and anchor three-dimensional actin filament networks with cell membranes or act as a scaffolding protein (29). FLNc is a muscle-specific isoform that has been identified as a substrate of Akt (29) that can be phosphorylated on Ser2213 in response to insulin (11). In the current study, insulin-stimulated pFLNc^{Ser2213} of isolated soleus muscles was enhanced with CR. This result

is similar to our previous reports in epitrochlearis muscles from 9-mo-old rats (35). The functional role of insulin-mediated FLNc phosphorylation remains to be elucidated, but our data identify FLNc as an Akt substrate that is responsive to both CR and insulin in the predominantly slow-twitch soleus muscle.

We also measured phosphorylation of other important insulin-responsive Akt substrates. As expected, we found that a physiological insulin dose was able to induce site-specific phosphorylation of GSK3α^{Ser21} and GSK3β^{Ser9}, serine/threonine kinases that have diverse cellular functions (6, 41); TSC2^{Ser939}, a protein with an important role in the control of cell size (23); and pP70S6K^{Thr412}, a serine/threonine kinase that is key regulator of protein synthesis (44). Our novel observations included a significant CR effect on pP70S6K^{Thr412} and strong trends ($P = 0.06$) for CR effects on pGSK3β^{Ser9} and pTSC2^{Ser939}, along with no evidence for a CR effect on pGSK3α^{Ser21}. Together with the results for pAS160^{Thr642} and pFLNc^{Ser2213}, these data clearly demonstrate that CR has a nonuniform influence on several key Akt substrates in rat soleus muscles.

Our group has previously observed that CR effects on insulin signaling are not identical in all skeletal muscles. McCurdy et al. (25) found that CR led to increased insulin-stimulated glucose uptake in both the predominantly fast-twitch extensor digitorum longus (EDL) and the soleus muscles of wild-type mice. In Akt2-null mice, the CR effect was eliminated completely in the EDL, but only partly reduced in the soleus of male mice, suggesting a possible role for an Akt2-independent mechanism only in the soleus. We have also studied 9-mo-old rats and found that despite no diet effects on either pIR or IRS1-associated PI3K activity with a submaximally effective insulin dose, Akt phosphorylation was increased for CR versus AL groups in both epitrochlearis and soleus, concomitant to an increase in glucose uptake. However, a CR-induced increase in pAS160 was observed in the epitrochlearis but not in the soleus (34). The results of the current study are consistent with our earlier study of 9-mo-old rats (34), which also found a significant diet effect of pAkt (CR > AL) on insulin-stimulated Akt phosphorylation concomitant with an increase in glucose uptake, despite no increases pIR$^{Tyr1162/1163}$ or pAS160^{Thr642}.

In summary, the current study extends earlier research by demonstrating that the CR-induced increase in pAkt in isolated soleus muscles from 9-mo-old rats can occur without concomitant enhancement of several important insulin signaling events that are proximal to Akt activation, including pIR$^{Tyr1162/1163}$, pIRS1^{Tyr}, pIRS1^{Ser312}, IRS1-associated PI3K activity, or pPTEN^{Ser380}. These results suggest that the greater pAkt in the soleus muscles from CR rats were attributable to an alternative mechanism. Our working hypothesis is that the improved Akt activation with CR is related to altered Akt binding to protein partners that regulate Akt phosphorylation. It will be important for future research to test this idea by evaluating Akt binding to proteins that have been reported to favor greater Akt phosphorylation [e.g., PHLDB1 (45) and ClipR-59 (12)] as well Akt's binding to carboxy-terminal modulator protein (CTMP) (14) and Tribble 3 (TRB3) (13) that have been reported to favor less pAkt. The signaling link between Akt and glucose uptake accounting for CR's ability to elevate insulin-stimulated glucose uptake remains to be determined, as CR did not enhance insulin-stimulated pAS160^{Thr642} but did lead to greater insulin-stimulated pFLNc^{Ser2213} in the soleus. Notably,

CR effects were not uniform for phosphorylation of six important insulin-regulated Akt substrates in the soleus. This result, using a physiologically relevant dietary model, is consistent with other studies that have used other experimental approaches (e.g., Akt inhibitors or gene knockouts) in cultured cells to probe the degree to which modulating Akt activation results in changes in phosphorylation of various Akt substrates (39). The differential response that we observed for CR effects on phosphorylation of Akt substrates likely has important implications for explaining complex effects of CR on diverse cellular functions.

AUTHOR CONTRIBUTIONS

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

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