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Inhibition of Akt2 phosphorylation abolishes the calorie restriction-induced improvement in insulin-stimulated glucose uptake by rat soleus muscle

Naveen Sharma[1,4], **Edward B. Arias**[1], and **Gregory D. Cartee**[1,2,3]

[1]Muscle Biology Laboratory, School of Kinesiology, University of Michigan, Ann Arbor, MI, USA

[2]Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA

[3]Institute of Gerontology, University of Michigan, Ann Arbor, MI, USA

[4]School of Health Sciences, Central Michigan University, Mount Pleasant, MI, USA

Abstract

Calorie restriction (CR; ~60–65% of *ad libitum*, AL, consumption) can enhance insulin-stimulated glucose uptake (ISGU) in predominantly slow-twitch skeletal muscles (e.g., soleus) by an incompletely understood mechanism. We used an Akt inhibitor (MK-2206) to eliminate CR's effect on insulin-stimulated Akt2 phosphorylation in isolated rat soleus muscles. We found long-term CR-enhanced ISGU was abolished by eliminating the CR-effect on Akt2 phosphorylation, suggesting the CR-induced benefit on ISGU in the predominantly slow-twitch soleus relies on enhanced Akt2 phosphorylation.

Keywords

glucose transport; insulin signaling; dietary restriction; slow-twitch muscle; protein kinase B

INTRODUCTION

Skeletal muscle, a heterogeneous tissue composed of multiple fiber types, is the major tissue for insulin-stimulated glucose disposal (DeFronzo 1988). A well-described health-related benefit of calorie restriction (CR; ~60% of *ad libitum*, AL, consumption) is improved insulin sensitivity (McCurdy et al. 2005; Sharma et al. 2012c; Sharma et al. 2012b). Insulin-stimulated glucose uptake (ISGU), which is enhanced with CR (McCurdy et al. 2005; Sharma et al. 2011; Sequea et al. 2012; Sharma et al. 2012a; Sharma et al. 2015; Wang et al. 2016b), is attributed to enhanced activation of the serine/threonine kinase Akt (McCurdy et al. 2003; McCurdy et al. 2005; Sharma et al. 2011; Sequea et al. 2012; Sharma et al. 2012a; Sharma et al. 2012c; Sharma et al. 2012d; Sharma et al. 2015; Wang et al. 2016a; Wang et al. 2016b). Consistent with the fact that Akt2 is the major Akt isoform for ISGU (Bae et al.

Correspondence: Naveen Sharma, Ph.D., Central Michigan University, School of Health Sciences, Room 1174, Mount Pleasant, MI, 48859, Phone: (989) 774-2927, Fax: (989) 774-2908, sharm2n@cmich.edu.

Conflict of interest statement

The authors declare that there are not conflicts of interest.

2003; Sakamoto et al. 2006), previous studies demonstrated that CR increases insulin-stimulated Akt2 activation (McCurdy et al. 2003; McCurdy et al. 2005; Sequea et al. 2012; Sharma et al. 2012a; Wang et al. 2016b).

There are muscle-specific differences in CR-effects. In male Akt2-knockout (Akt2-KO) mice, Akt2 is essential for the CR-effect on ISGU in the predominantly fast-twitch extensor digitorum longus (EDL) (McCurdy et al. 2005). In contrast, ISGU of the soleus (largely slow-twitch) from the same Akt2-KO mice was greater for the CR versus AL group. A caveat of this study was that Akt2 expression was eliminated in all tissues throughout the development of Akt2-KO animals. Subsequently, we studied isolated rat epitrochlearis (predominantly fast-twitch) muscles (Sharma et al. 2012a; Wang et al. 2016a) incubated with a selective Akt-inhibitor, MK-2206 (Hirai et al. 2010; Tan et al. 2011), at a dose that reduced Akt2 phosphorylation of insulin-treated muscles from CR rats to levels similar to AL muscles. Preventing the CR-induced increase in Akt2 phosphorylation eliminated the CR-effect on epitrochlearis ISGU. We now extend this research by evaluating the Akt-inhibitor MK-2206's effect on insulin-stimulated Akt2 phosphorylation and glucose uptake of isolated soleus from CR and AL rats. We hypothesized that preventing CR's effect on Akt2 phosphorylation in the soleus would reduce ISGU in the CR group.

MATERIALS AND METHODS

Materials for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Hercules, CA). Anti-phospho Akt Thr^{308} ($pAkt^{Thr308}$), anti-phospho Akt Ser^{473} ($pAktSer^{473}$), and anti-rabbit IgG horseradish peroxidase conjugate were from Cell Signaling Technology (Danvers, MA, USA). Anti-Akt2 was from R&D Biosystems (Minneapolis, MN, USA). Anti-phospho $AS160^{Thr642}$ ($pAS160^{Thr642}$) was from B-Bridge International (Cupertino, CA, USA). Anti-AS160 and anti-sheep IgG horseradish peroxidase conjugate were from EMD Millipore (Billerica, MA, USA). Phospho-Akt antibodies against the Thr308 and Ser473 sites recognize Thr309 and Ser474 on Akt2, respectively. MK-2206 was from Selleck Chemicals (Houston, TX, USA). [^{3}H]-2-Deoxy-D-glucose ([^{3}H]-2-DG) and [^{14}C]-mannitol were from PerkinElmer (Boston, MA, USA).

Animal care procedures were approved by the University of Michigan Committee on Use and Care of Animals. Male Fisher-344xBrown Norway rats were obtained from the National Institute on Aging (NIA) Calorie Restricted Rodent Colony. Data from these rats on CR-effects in the predominantly fast-twitch epitrochlearis have been published (Sharma et al. 2012a). Calorie restriction was initiated at 14weeks-old in CR rats (AL rats had free access to NIH31 chow; CR: NIH31/NIA fortified chow at ~60–65% of AL), until euthanization at ~9 months-old (CR duration of ~25 weeks).

Muscle dissection and incubation have been described (Sharma et al. 2011; Sharma et al. 2012a). Soleus strips were incubated (30 min; gassed with 95% O_2/5% CO_2 at 35 °C) in vials containing 2 ml Krebs Henseleit Buffer (KHB) supplemented with 0.1% bovine serum albumin (BSA), 2 mmol/L sodium pyruvate, 6 mmol/L mannitol, and DMSO or MK-2206 (0.1 μmol/L). Muscles were transferred to vials containing the identical solution as the previous step, ±1.2 nmol/L insulin (30 min). Muscles were transferred to a vial containing 2

ml KHB/BSA, the same concentration of MK-2206 and insulin as the previous step, 1mM 2-DG (2.25 mCi/mmol [^{3}H]-2-DG), and 9 mmol/L mannitol (0.022 mCi/mmol [^{14}C]-mannitol) for 20 min. Muscles were blotted, trimmed, freeze-clamped, and stored (−80°C). Muscles were homogenized and processed for immunoprecipitation and immunoblotting (Sharma et al. 2011; Sharma et al. 2012a), and [^{3}H]-2-Deoxy-D-glucose (2-DG) uptake (Hansen et al. 1994). One-way ANOVA was used for statistical comparison (SigmaPlot version 11.0; San Jose, CA). Data are mean ±SEM. A P value <0.05 was statistically significant.

RESULTS

Immunoblotting

There was no significant diet-related difference for muscles incubated without insulin and MK-2206 for pan-Akt Thr^{308}-phosphorylation (Fig. 1A), Akt2 Thr^{309}-phosphorylation (Fig. 1B), pan-Akt Ser^{473}-phosphorylation (Fig. 1C), or Akt2 Ser^{474}-phosphorylation (Fig. 1D). For insulin-stimulated muscles without MK-2206, phosphorylation of Akt^{Thr308}, Akt2^{Thr309}, Akt^{Ser473} and Akt2^{Ser474} of CR exceeded (P<0.05) AL. Incubation of insulin-stimulated CR muscles with MK-2206 reduced (P<0.05) pan-Akt^{Thr308}, Akt2^{Thr309}, pan-Akt^{Ser473} and Akt2^{Ser474} phosphorylation versus insulin-stimulated CR muscles without MK-2206. Pan-Akt^{Ser473} phosphorylation was significantly (P<0.05) reduced in insulin-stimulated CR muscles with MK-2206 versus AL insulin-stimulated muscles (Fig. 1C). There was no significant diet-related difference for muscles incubated without insulin and MK-2206 for AS160 Thr^{642}-phosphorylation (Fig. 2A). There were also no differences among insulin-stimulated groups for AS160 Thr^{642}-phosphorylation. There were no group differences for total Akt2 or AS160 abundance (data not shown).

2-DG Uptake

There was no significant diet-related difference in 2-DG uptake for muscles without insulin and MK-2206 (Fig. 2B). For insulin-stimulated muscles without MK-2206, 2-DG uptake of CR exceeded (P<0.05) AL. Incubation of insulin-stimulated CR muscles with MK-2206 reduced (P<0.05) 2-DG uptake versus insulin-stimulated CR muscles without MK-2206. 2-DG uptake was not significantly different in insulin-stimulated AL muscles versus insulin-stimulated CR muscles with MK-2206.

DISCUSSION

We were successful in using a selective Akt-inhibitor to eliminate the CR-induced increase in pan-Akt and Akt2 phosphorylation on each of the sites important for enzyme activity and increasing ISGU. Rapid Akt2 inhibition avoided the caveats associated with the life-long, whole body Akt2-KO model used previously (McCurdy et al. 2005). Together with the concomitant MK-2206-associated elimination of the CR-induced increase in ISGU, the current data provides compelling new evidence that insulin-stimulated Akt2 phosphorylation is essential for the CR-induced enhancement in ISGU by the predominantly slow-twitch rat soleus. The current results in the soleus are similar to results for the predominantly fast-twitch rat epitrochlearis using MK-2206 in the same rats (Sharma et al. 2012a). These

results support the idea that, at least in rats, long-term CR relies on an Akt2-dependent mechanism for improving ISGU by both slow-twitch and fast-twitch muscles.

McCurdy et al. used Akt2-KO mice to probe Akt2's role in CR-induced increase in ISGU (McCurdy et al. 2005). The magnitude of ISGU in the soleus of male CR mice was reduced versus wild-type CR mice, but a significant CR-effect on ISGU was found in Akt2-KO animals, suggesting a possible Akt2-independent mechanism. The differing results in the current study versus the earlier study might be related to species differences or compensatory mechanisms with whole-body elimination of Akt2 in Akt2-KO mice.

During a euglycemic-hyperinsulinemic clamp, CR increased glucose uptake by several predominantly fast-twitch muscles, but it did not significantly increase glucose uptake by the soleus (Sharma et al. 2014). The isolated muscle model provides valuable information about mechanisms that are inherent to the muscle itself, whereas the clamp results reflect both muscle and systemic (vascular, endocrine and neural) mechanisms. In the soleus of AL animals during a clamp, there is evidence that glucose transport across the sarcolemma is not a rate-limiting step, whereas it is rate-limiting in fast-twitch muscles (Halseth et al. 2001; Petersen et al. 2003). Other experimental differences may have contributed to the divergent results, including the duration and concentration for insulin exposure in the clamp (145 min with ~0.84 nmol/L) versus the isolated soleus (50 min with 1.2 nmol/L).

AS160 is the Akt2 substrate that has been most convincingly linked to increased ISGU (Sano et al. 2003; Cartee 2015). In the rat epitrochlearis, CR produces increased phosphorylation of AS160 (Sharma et al. 2011; Sharma et al. 2012a). However, insulin-stimulated AS160 phosphorylation was not enhanced above AL controls in the soleus in this study or an earlier study of 9 month-old rats undergoing the same CR protocol (Sharma et al. 2011; Sharma et al. 2012d). These results suggest CR might rely on greater phosphorylation of Akt2 substrates besides AS160 in the soleus. It would also be valuable to determine if CR alters AS160's subcellular localization and/or binding to regulatory proteins which modify AS160 function (Stockli et al. 2011; Tan et al. 2012). The current data support the idea that the CR-effect on Akt2 is necessary for the CR-improvement in ISGU, but it uncertain if greater Akt2 activation is sufficient for CR-induced elevation in ISGU. It is possible that CR-effects on ISGU require additional changes in Akt2-independent processes.

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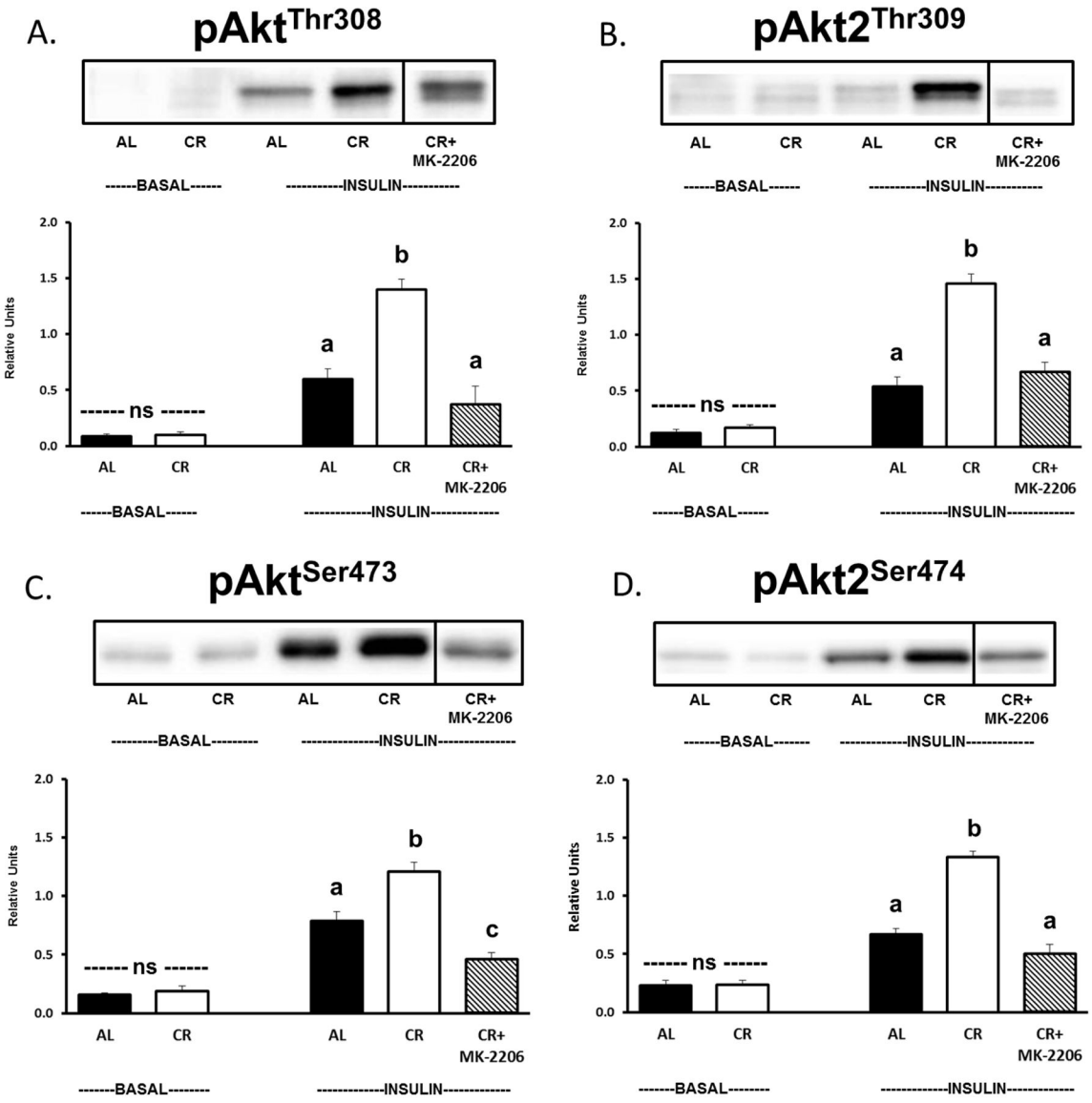

Figure 1.

Immunoblot analysis of Akt phosphorylation in rat soleus muscle. (A) pan-Akt Thr308 phosphorylation (pAkt^{Thr308}). (B) Akt2 Thr309 phosphorylation (pAkt2^{Thr309}), muscle lysates were immunoprecipitated with Akt2 antibody prior to immunoblotting with phospho-Akt^{Thr308} antibody, (C) pan-Akt Ser473 phosphorylation (pAkt^{Ser473}), and (D) Akt2 Ser474 phosphorylation (pAkt2^{Ser474}), muscle lysates were immunoprecipitated with Akt2 antibody prior to immunoblotting with phospho-Akt^{Ser473} antibody. Filled bars are muscles from AL-fed rats, open bars are muscles from CR-fed rats, and hatched bar are muscles from CR-fed rats incubated with MK-2206. “ns” indicates no statistical difference between AL and CR groups in the absence of insulin. Insulin-stimulated groups with matching letters are not statistically different, and groups with differing letters are statistically ($P < 0.05$) different from each other. Values are means ± SE, n = 6–10 per treatment group. All lanes are from

the same gel, however, the representative blots have a vertical line separating the final 2 lanes indicating that the original blot has been re-ordered to match the respective bar graphs.

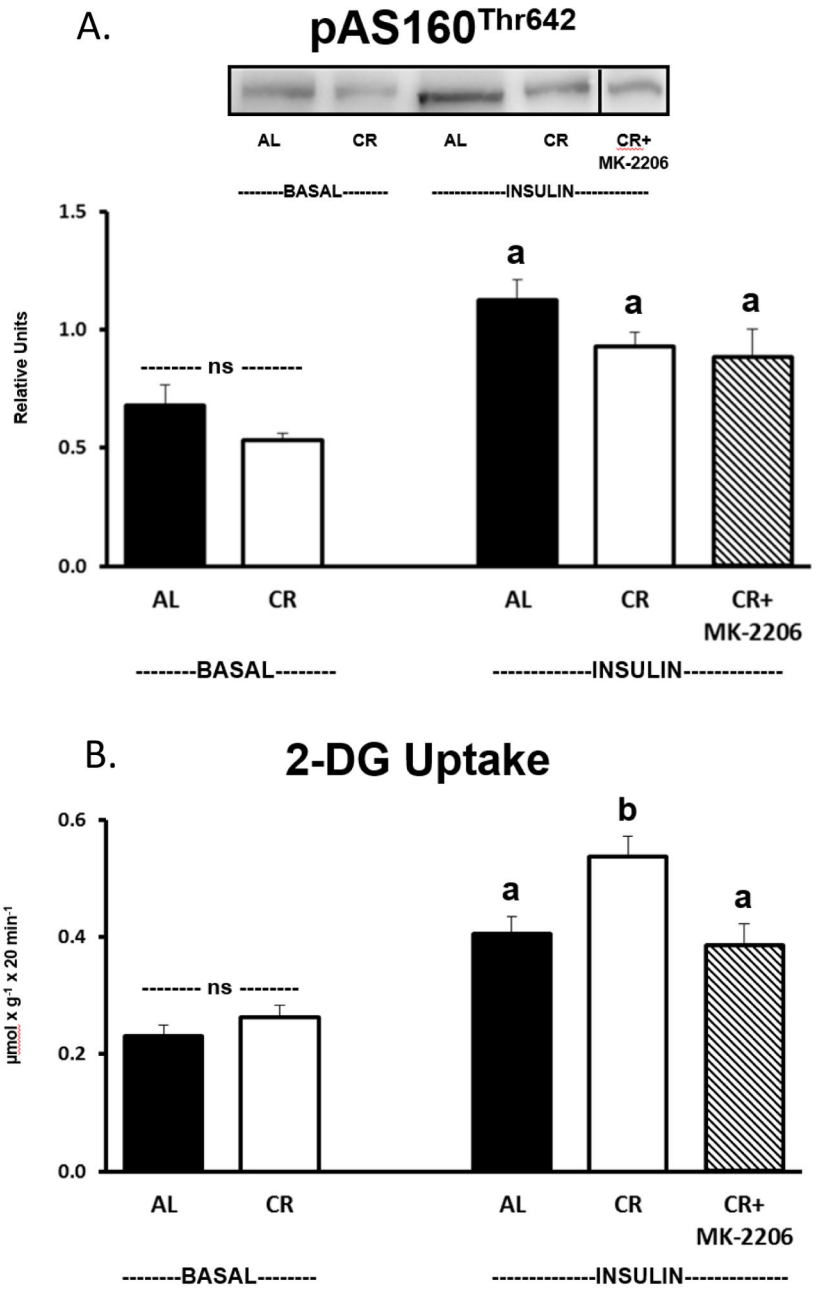

Figure 2.

AS160 Thr^{642}-phosphorylation (pAS160^{Thr642}) and 2-deoxy-D-glucose (2-DG) uptake in rat soleus muscle. (A) pAS160^{Thr642}. (B) 2-DG uptake. Filled bars are muscles from AL-fed rats, open bars are muscles from CR-fed rats, and hatched bar are muscles from CR-fed rats incubated with MK-2206. “ns” indicates no statistical difference between AL and CR groups in the absence of insulin. Insulin-stimulated groups with matching letters are not statistically different, and groups with differing letters are statistically ($P < 0.05$) different from each other. Values are means ± SE, n = 12–18 per treatment group.