

Akt2 Is Essential for the Full Effect of Calorie Restriction on Insulin-Stimulated Glucose Uptake in Skeletal Muscle

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Brief calorie restriction (CR; 20 days of 60% of ad libitum [AL] intake) improves insulin-stimulated glucose transport, concomitant with enhanced phosphorylation of Akt2. The purpose of this study was to determine whether Akt2 is essential for the calorie restriction-induced enhancement in skeletal muscle insulin sensitivity. We measured insulin-stimulated 2-deoxyglucose (2DG) uptake in isolated extensor digitorum longus (EDL) and soleus muscles from male and female wild-type (WT) and Akt2-null (knockout [KO]) mice after ad libitum or calorie-restricted (20 days at 60% of AL) feeding. In WT mice, calorie restriction significantly enhanced insulin-stimulated 2DG uptake in both muscles regardless of sex. However, in KO mice, calorie restriction did not enhance insulin-stimulated 2DG in male or female EDL or in female soleus. Only in male KO soleus did calorie restriction significantly increase insulin-stimulated 2DG through an Akt2-independent mechanism, although 2DG uptake of the KO-CR group was reduced compared with the WT-CR soleus group. Akt2 serine phosphorylation was enhanced approximately two- to threefold in insulin-stimulated WT-CR versus WT-AL muscles. Calorie restriction induced an ~1.5- to 2-fold elevation in Akt1 phosphorylation of insulin-treated muscles, regardless of genotype, but this increase was insufficient to replace Akt2 for insulin-stimulated 2DG in Akt2-deficient muscles. These results indicate that Akt2 is essential for the full effect of brief calorie restriction on insulin-stimulated glucose uptake in skeletal muscle with physiologic insulin. *Diabetes* 54: 1349–1356, 2005

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2DG, 2-deoxyglucose; AMPK, AMP-activated protein kinase; EDL, extensor digitorum longus; IRS, insulin receptor substrate; KHB, Krebs-Henseleit buffer; PI3K, phosphatidylinositol 3-kinase; TBST, Tris-buffered saline with 0.1% Tween.

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Moderate calorie restriction (25–40% reduction below ad libitum intake) is an effective treatment for improving insulin sensitivity in many species (1–4). Improved insulin sensitivity occurs rapidly (manifested within 20 days of calorie restriction) (1,3,4). In skeletal muscle, brief calorie restriction (5–20 days) enhances insulin-stimulated glucose uptake (5), a rate-limiting step in glucose metabolism. This increase is ultimately due to an increase in the translocation of GLUT4 glucose transporter to the cell surface after insulin stimulation (6). However, the specific cellular mechanism(s) leading to calorie-restricted enhancement of insulin-stimulated GLUT4 translocation is uncertain.

Phosphatidylinositol 3-kinase (PI3K) activation is essential for insulin-stimulated glucose transport (7,8) and is therefore a possible mediator of the effects of calorie restriction on insulin sensitivity. Although we have repeatedly seen a trend for increased insulin receptor substrate (IRS)-1-associated PI3K activity, we have not found a significant change in IRS-1-, IRS-2-, or phosphotyrosine-associated PI3K activity (6,9,10). There is no change in insulin receptor number, binding affinity, or tyrosine kinase activity with brief calorie restriction (11,12). There is also no increase in IRS-1 or IRS-2 (4,6) or PI3K regulatory p85 subunit (6) abundance. Considering the lack of a significant effect of calorie restriction on PI3K and its upstream regulators, we have become interested in insulin signaling downstream of PI3K.

Akt is an insulin-responsive serine/threonine kinase downstream of PI3K that is important for insulin-stimulated glucose uptake (13–15). There are three Akt isoforms (Akt1, Akt2, and Akt3) that have distinct but overlapping signaling roles. Akt2 is of particular interest, as it plays a key role in insulin-stimulated glucose uptake (14,16,17). Specifically, isoform-targeted knockdown of Akt2 using siRNA significantly reduced insulin-stimulated glucose uptake, while almost complete knockdown of Akt1 had much less effect (13,17). Moreover, Akt2-null mice demonstrate impaired glucose tolerance and reduced insulin-stimulated glucose uptake (18,19), while Akt1-null mice have normal glucoregulation (20). However, the impairment in skeletal muscle glucose uptake of Akt2-null mice is incomplete, as it can be overcome at high insulin concentrations, suggesting functional redundancy within Akt isoforms or a compensatory pathway(s). Importantly, reduced Akt2 expression and impaired insulin-stimulated Akt2 activation were

reported for diabetic (21) and insulin-resistant (22) human skeletal muscle.

Recently, we found that 20 days of calorie-restricted compared with ad libitum feeding increased the amount of insulin-stimulated Akt2 phosphorylation but not the amount of Akt1 phosphorylation in rat epitrochlearis muscle (23). These data suggest an important role for Akt2 in the calorie restriction-induced increase in insulin sensitivity. Therefore, the primary aim of the present study was to determine whether Akt2 is essential for the calorie restriction-induced increase in insulin-stimulated glucose uptake. To investigate this question, we measured 2-deoxyglucose (2DG) uptake in isolated skeletal muscles after 20 days of calorie-restricted or ad libitum feeding in male and female mice homozygous wild-type (WT) or null (knockout [KO]) for the Akt2 allele. To further elucidate the mechanism(s) mediating changes in 2DG uptake with calorie restriction, phosphorylation and abundance of Akt1 and Akt2 were measured. Our results indicate that Akt2 is required for the full effect of brief calorie restriction on insulin-stimulated glucose uptake in mouse skeletal muscle at a physiological insulin concentration.

RESEARCH DESIGN AND METHODS

Dietary treatment. Male mice null for Akt2 on C57BL/6 background (18) were bred to female C57BL/6 mice (Charles River, Wilmington, MA) to produce mice heterozygous for the null and intact Akt2 alleles. Heterozygous mice were mated to produce littermates that were homozygous for the intact Akt2 allele (WT) and heterozygous and homozygous for the null Akt2 allele (KO). PCR analysis from tail-tip DNA was performed to identify genotype.

Animal care was approved by University of Wisconsin-Madison Animal Care and Use Committee. Mice were maintained on a 12:12-h light-dark cycle (0600:1800) with ad libitum access to food (Teklad Rodent Diet 8604; Harlan Teklad, Madison, WI) and water before the study. At 7–10 weeks old, WT and KO mice were housed in individual wire-bottomed cages with free access to precision pellet diet (Bio-Serv, Frenchtown, NJ) and water for a 7-day acclimation period. Baseline food consumption was then measured for 7 days, and individual baseline food intake was calculated. Male and female WT and KO mice were randomly assigned to ad libitum (AL) control or calorie-restricted (CR) groups (male/female WT-CR, WT-AL, KO-CR, or KO-AL) for 20 days. CR mice received 60% of baseline intake at 1700, while AL mice had daily free access to food. All mice had free access to water. On the day of the muscle experiment (0730), all mice were fasted with access to water. At 0900, blood was drawn from unanesthetized mice by maxillary bleed as described by G.L. Sottacasa (available at www.univ.trieste.it/~servpoli/stabul.htm). From 1000–1200, mice were anesthetized (intraperitoneal injection of sodium pentobarbital, 50 mg/kg). After loss of pedal reflex, soleus and EDL were dissected for *in vitro* incubation. One retroperitoneal fat pad per animal was dissected and weighed.

In vitro incubation. To measure 2DG uptake, paired soleus and EDL were incubated at 37°C for 30 min in oxygenated (95% O₂, 5% CO₂) flasks of Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 2 mmol/l Na-pyruvate, and 6 mmol/l mannitol, with one muscle per pair in KHB without insulin and the contralateral muscle in KHB with 0.6 nmol/l insulin. After 30 min, muscles were transferred to a second flask and incubated at 37°C for 20 min in KHB plus 0.1% BSA, 9 mmol/l [¹⁴C]-mannitol (0.053 mCi/mmol; Amersham Pharmacia, Piscataway, NJ), and 1 mmol/l [³H]-2DG (6 mCi/mmol; Perkin Elmer, Boston, MA) with the same insulin concentration as in the first incubation. After 20 min, muscles were blotted on ice-cold filter paper, trimmed and freeze-clamped, and then stored (–80°C). 2DG uptake rate was calculated as previously described (24).

Muscle preparation. Frozen muscles were weighed and homogenized as previously described (23). The homogenate was transferred to microfuge tubes and solubilized (1–2 h, 4°C) with end-over-end rotation. The homogenate was centrifuged (12,000g, 12 min, 4°C). Aliquots of supernatant were frozen (–80°C) for immunoblotting or used immediately for 2DG uptake measurement. Total protein concentration of supernatant was determined by bicinchoninic acid assay (Pierce, St. Louis, MO) (25).

Serum analysis. Collected blood was allowed to clot at room temperature for 30 min in microserum tubes and centrifuged (300g, 15 min, 4°C). Aliquots of serum were stored (–80°C). Serum glucose was determined by colorimetric

glucose oxidase assay (ThermoDMA, Louisville, CO). Serum insulin was measured by rat sensitive radioimmunoassay (Linco Diagnostics, St. Louis, MO).

Immunoprecipitation. For Akt1/Akt2 immunoprecipitation, 2 µg antibody (goat anti-Akt1, catalog no. sc-7126; Santa Cruz Biotechnology, Santa Cruz, CA, or rabbit anti-Akt2 antibody, no. 2962; Cell Signaling, Beverly, MA) was incubated with supernatant (2–3 h/overnight, 4°C) with rotation. After initial antibody incubation, protein G/A agarose beads (Upstate Biotechnology, Lake Placid, NY), prewashed in immunoprecipitation buffer no. 1 (50 mmol/l PBS, 1% Igepal, 0.1 mmol/l Na₃VO₄), were added to the lysate/antibody mix and rotated (4°C overnight/3 h). Beads were washed six times: three times in immunoprecipitation buffer no. 1 and three times in immunoprecipitation buffer no. 2 (10 mmol/l Tris pH 7.5, 100 mmol/l NaCl, 0.1 mmol/l Na₃VO₄) and eluted with 2 × SDS buffer.

Immunoblot assay. For serine phosphorylation of Akt isoforms, immunoprecipitants were separated on 8% SDS-PAGE and transferred to nitrocellulose at 200 mA for 2 h at 4°C. Membranes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween (TBST) at room temperature and then incubated (1:1,000) with anti-phosphoserine Akt (catalog no. 9271; Cell Signaling, Beverly, MA) overnight at 4°C. For Akt1 abundance, membranes were blocked overnight with 5% milk-TBST and then incubated with anti-Akt1 (1:500). For Akt2 abundance, membranes were blocked in milk-TBST and then incubated with anti-Akt2 (1:1,000) overnight at 4°C. For threonine phosphorylation of AMP-activated protein kinase (AMPK), membranes were blocked in milk-TBST and then incubated with anti-phosphothreonine AMPK-α (catalog no. 2531; Cell Signaling) at 1:1,000 overnight (4°C). After secondary antibody incubations, blots were developed by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and bands were quantitated by densitometry (Bio-Rad, Hercules, CA). Band densities were expressed relative to insulin-stimulated WT-AL group mean for Akt phosphorylation data or WT-AL group mean for AMPK phosphorylation and Akt abundance data, which were adjusted to equal 1.0 on each blot.

Statistical analysis. Two-way ANOVA with genotype and diet as main effects was used to determine significant differences within each sex for all measurements except Akt2 phosphorylation and abundance. 2DG uptake data were analyzed for each insulin level (0 and 0.6 nmol/l) and for calculated insulin-stimulated 2DG uptake. Calculated insulin-stimulated 2DG uptake equals the insulin 2DG uptake minus the basal 2DG uptake for paired muscles and represents the insulin effect on glucose uptake without contribution of basal levels. Data that failed the Levine Normality test were log transformed and analyzed by two-way ANOVA. Tukey post hoc test was applied to determine the source of significant variance. For Akt2 serine phosphorylation, Akt2 abundance, and AMPK phosphorylation, Student's *t* test was used. *P* < 0.05 was considered significant.

RESULTS

Body and fat pad weight. On the first day of the calorie restriction period, initial body weight was not different between AL and CR groups with the same genotype and sex. However, all KO groups were ~8% smaller than their sex- and diet-matched WT controls (Table 1). After 20 days, the ~8% reduced body size persisted only in the male KO-AL group compared with the WT controls (*P* < 0.01). Final body weight was decreased for calorie restriction versus ad libitum regardless of genotype or sex.

KO-AL versus WT-AL mice had significantly smaller retroperitoneal fat pads regardless of sex (males ~60%, females ~40%). Retroperitoneal fat pad weight was significantly lower in the WT-CR versus the WT-AL group for both sexes (males ~90%, females ~35%; *P* < 0.05). In KO mice, retroperitoneal fat pad weight was ~75% lower with calorie restriction versus ad libitum feeding only in males (*P* < 0.05). Expressed relative to final body weight (milligrams per gram), the calorie restriction versus ad libitum reduction in fat pad weight was significant (*P* < 0.005) in male (WT = 80%, KO = 67%) but not female (WT = 30%, KO = 20%) mice, regardless of genotype.

Serum glucose and insulin. Fasting (~3-h) serum glucose and insulin were measured the morning of the muscle experiment (Table 2). Glycemia was not different between male WT-AL and KO-AL mice. However, serum glucose

TABLE 1
Body weight and retroperitoneal fat pad weight for WT and KO mice with ad libitum or calorie-restricted feeding

	WT		KO	
	Ad libitum	Calorie restriction	Ad libitum	Calorie restriction
Males				
Initial weight (g)	23.6 ± 0.7*	24.6 ± 0.5†	21.4 ± 0.6	22.5 ± 0.6
Final weight (g)	25.4 ± 0.8*†	17.7 ± 0.3*	23.3 ± 0.5*	17.3 ± 0.5
Fat pad (g)	0.171 ± 0.024*†	0.021 ± 0.004	0.073 ± 0.009*	0.019 ± 0.002
Females				
Initial weight (g)	19.6 ± 0.5†	19.8 ± 0.5†	17.8 ± 0.6	17.8 ± 0.4
Final weight (g)	20.7 ± 0.4*†	16.1 ± 0.5	19.9 ± 0.6*	14.8 ± 0.5
Fat pad (g)	0.063 ± 0.010*†	0.040 ± 0.007	0.038 ± 0.009	0.022 ± 0.003

Data are means ± SE. Initial (day 1 of calorie reduction) and final (day 20 of calorie reduction) body weight was measured ($n = 7-10$). Retroperitoneal fat pad was weighed after day 20 ($n = 7-9$). For each sex, data analyzed by two-way ANOVA for diet and genotype. Tukey post hoc analysis: * $P < 0.05$ AL > CR within genotype; † $P < 0.05$ WT > KO within diet.

was significantly reduced in male WT-CR versus WT-AL mice (~30%; $P < 0.01$) but not in KO-CR compared with KO-AL mice. In female mice, serum glucose was unaffected by diet or genotype. Serum insulin was not significantly different between WT-AL and WT-CR in either sex, although there was a trend for a calorie restriction-induced decline in WT males. However, serum insulin was ~11-fold higher in male KO-AL versus WT-AL mice ($P = 0.001$). Insulin was significantly reduced ($P = 0.004$) in male KO-CR versus KO-AL, and the KO-CR group attained levels similar to male WT-AL mice. There was a trend for approximately twofold higher insulin in female KO-AL versus WT-AL ($P = 0.09$). Unexpectedly, calorie restriction in KO females resulted in an approximately threefold increase in insulin compared with KO-AL ($P < 0.05$).

2DG uptake. In EDL muscles, basal 2DG uptake was significantly ($P < 0.001$) increased with calorie restriction compared with ad libitum feeding, regardless of genotype or sex (Fig. 1). In the soleus, calorie restriction significantly enhanced basal 2DG ($P = 0.01$) only in male WT-CR versus WT-AL (Fig. 2). With a physiologic insulin concentration, calorie restriction significantly enhanced 2DG uptake above ad libitum feeding in both the EDL and soleus of WT mice, regardless of sex (Figs. 1 and 2). In contrast, in KO mice, 2DG uptake with insulin was not significantly increased above ad libitum with calorie restriction in male or female EDL or in female soleus. Only in male KO soleus was 2DG uptake in the presence of insulin significantly increased by calorie restriction above ad libitum values. In all groups, 2DG uptake with insulin was ~1.5-fold greater for WT-CR versus KO-CR in the respective muscle from the same sex ($P < 0.05$).

Insulin-stimulated 2DG uptake represents the calculated

effect of insulin on muscle 2DG above basal 2DG uptake (insulin-stimulated 2DG uptake = 2DG uptake with insulin – basal 2DG uptake). Because basal glucose uptake was increased by calorie restriction in some muscles, it is important to consider changes in insulin-stimulated glucose uptake to isolate diet and/or genotype effects on insulin action from altered basal glucose uptake (Table 3). In WT mice, insulin-stimulated 2DG uptake was significantly ($P \leq 0.01$) increased in calorie restriction versus ad libitum in EDL (~60% for both sexes) and soleus (males ~56%, females ~40%) regardless of sex. However, in KO mice, calorie restriction did not enhance insulin-stimulated 2DG uptake in the EDL of either sex or the female soleus. In male soleus, insulin-stimulated 2DG uptake was enhanced in KO-CR versus KO-AL ($P = 0.02$). Notably, the insulin-stimulated 2DG uptake was always significantly ($P < 0.05$) and markedly (>1.6-fold) greater in WT-CR compared with KO-CR.

Akt1 serine phosphorylation. Akt1 abundance (data not shown, $n = 5-7$) was not significantly different for diet or genotype in EDL or soleus. Akt1 phosphorylation without insulin (Figs. 3 and 4) was not significantly different for diet or genotype in EDL or soleus from females or in EDL from males. For muscles without insulin, only the soleus from males had a significant ($P < 0.01$) calorie restriction-induced increase in Akt1(Ser⁴⁷³) phosphorylation (Fig. 4). In insulin-stimulated muscles, Akt1(Ser⁴⁷³) phosphorylation was significantly ($P < 0.05$) enhanced in calorie-restricted compared with ad libitum feeding in male and female EDL (25–90% increase) and male soleus (40–80% increase). In insulin-stimulated EDL, calorie-restricted compared with ad libitum feeding had higher Akt1 serine phosphorylation (~2-fold in males, ~1.4-fold in females).

TABLE 2
Serum insulin and glucose for WT or KO mice after ad libitum or calorie-restricted diet

	WT		KO	
	Ad libitum	Calorie restriction	Ad libitum	Calorie restriction
Males				
Insulin (ng/ml)	1.0 ± 0.2*	0.4 ± 0.1*	11.6 ± 13.3†	1.3 ± 0.2
Glucose (mmol)	8.8 ± 0.4†	6.2 ± 0.5*	7.8 ± 0.6	8.6 ± 0.4
Females				
Insulin (ng/ml)	0.5 ± 0.1	0.5 ± 0.1*	1.2 ± 0.4†	3.5 ± 0.8
Glucose (mmol)	8.2 ± 0.4	8.1 ± 0.5	8.0 ± 0.9	8.3 ± 1.2

Data are means ± SE ($n = 4-5$). Data were log transformed to normalize distribution and analyzed by two-way ANOVA for diet and genotype in each sex. Untransformed data are shown. Tukey post hoc analysis: * $P < 0.05$ WT < KO within diet; † $P < 0.05$ AL vs. CR within genotype.

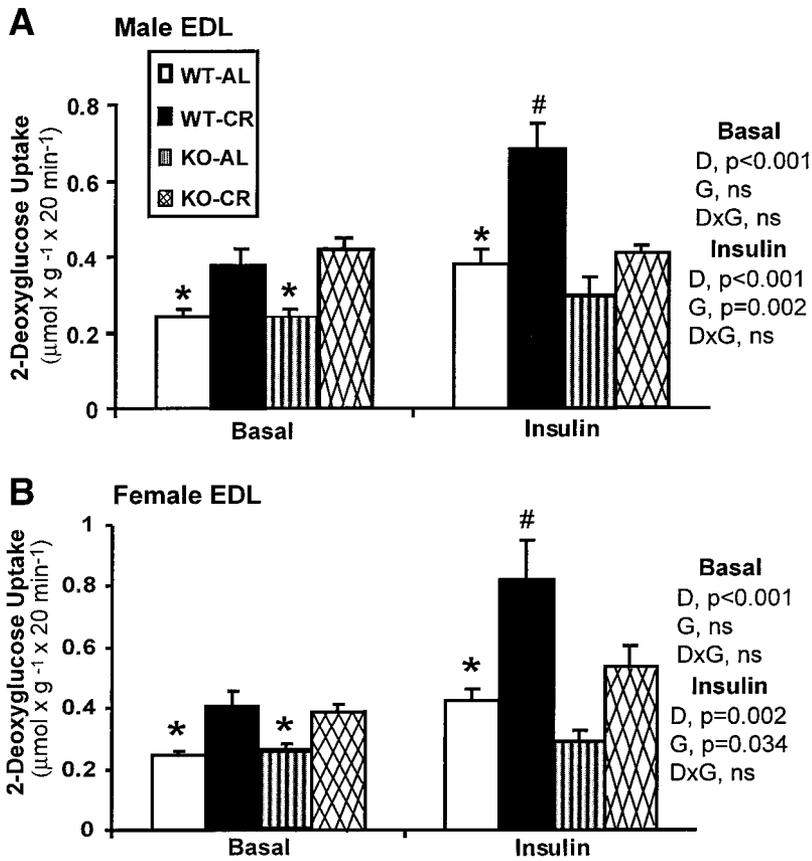


FIG. 1. 2DG uptake in EDL, without (basal) or with (0.6 nmol/l) insulin, from male (A) or female (B) WT and Akt2 KO mice after 20 days of ad libitum (AL) or calorie-restricted (CR) feeding. Data are means \pm SE ($n = 9-17$). Data for each sex were analyzed by two-way ANOVA within each insulin level. D, main effect of diet; G, main effect of genotype; D \times G, interaction between main effects. Tukey post hoc analysis was performed for a significant main effect. * $P < 0.05$ AL vs. CR within genotype; # $P < 0.05$ WT vs. KO within dietary group.

In insulin-stimulated male soleus, Akt1 phosphorylation was ~ 1.5 -fold greater in WT-CR versus WT-AL and ~ 2 -fold greater for KO-CR versus KO-AL. In female soleus, there was a trend ($P = 0.08$) for higher Akt1 phosphorylation in

insulin-stimulated muscles from CR versus AL mice, regardless of genotype.

Akt2 serine phosphorylation. Akt2 abundance (data not shown, $n = 6-9$) and Akt2 serine phosphorylation without

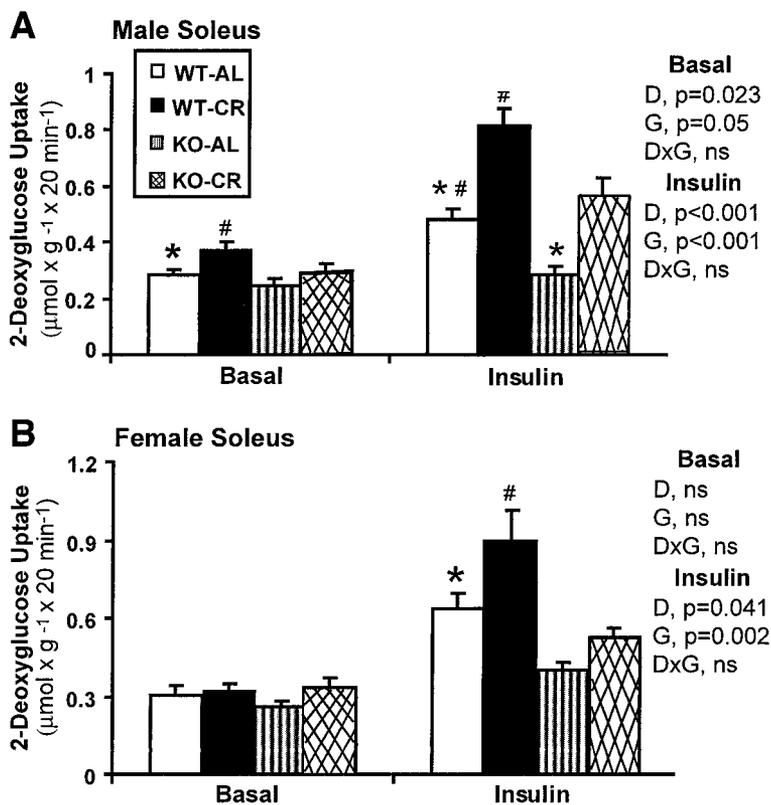


FIG. 2. 2DG uptake in soleus, without (basal) or with (0.6 nmol/l) insulin, from male (A) or female (B) WT and Akt2 KO mice after 20 days of ad libitum (AL) or calorie-restricted (CR) feeding. Data are means \pm SE ($n = 9-17$). Data for each sex were analyzed by two-way ANOVA within each insulin level. D, main effect of diet; G, main effect of genotype; D \times G, interaction between main effects. Tukey post hoc analysis was performed for a significant main effect. * $P < 0.05$ AL vs. CR within genotype; # $P < 0.05$ WT vs. KO within diet. There was a trend ($P = 0.09$) for female soleus with insulin, AL-WT $>$ AL-KO.

TABLE 3
Insulin-stimulated 2DG (2DG uptake with insulin minus basal 2DG uptake)

	WT		KO	
	Ad libitum	Calorie restriction	Ad libitum	Calorie restriction
Males				
EDL ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)	0.137 \pm 0.04	0.309 \pm 0.06*†	0.054 \pm 0.05	-0.008 \pm 0.03
Soleus ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)	0.197 \pm 0.04	0.448 \pm 0.07*†	0.036 \pm 0.05	0.269 \pm 0.07*
Females				
EDL ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)	0.174 \pm 0.04	0.416 \pm 0.09*†	0.027 \pm 0.03	0.148 \pm 0.04
Soleus ($\mu\text{mol} \cdot \text{g}^{-1} \cdot 20 \text{ min}^{-1}$)	0.340 \pm 0.04	0.582 \pm 0.04*†	0.147 \pm 0.04	0.192 \pm 0.05

Data are means \pm SE ($n = 9-15$). Values represent the effect of insulin on 2DG uptake above basal. For each sex, data are analyzed by two-way ANOVA for diet and genotype. Tukey post hoc analysis: * $P < 0.05$ AL < CR with same genotype; † $P < 0.05$ WT > KO within diet.

insulin (Fig. 5) were not significantly different for ad libitum versus calorie restriction in EDL or soleus of WT mice, regardless of sex. Without exception, Akt2 phosphorylation in insulin-stimulated muscle from WT mice was significantly higher for calorie-restricted compared with ad libitum feeding. In insulin-stimulated EDL of WT mice (Fig. 5A and E), Akt2(Ser⁴⁷⁴) phosphorylation was significantly increased for calorie restriction versus ad libitum in males (~2-fold) and females (~1.8-fold). Similarly, in insulin-stimulated WT soleus (Fig. 5B and D), Akt2(Ser⁴⁷⁴) phosphorylation

was significantly enhanced for calorie restriction versus ad libitum in males (~3-fold) and females (~2.5-fold).

AMPK threonine phosphorylation. AMPK(Thr¹⁷²) phosphorylation was evaluated in the EDL of male mice ($n = 6-9$) to determine whether the calorie restriction-induced increase in basal 2DG uptake was accompanied with a calorie restriction-induced increase in AMPK phosphorylation (AL-WT = 1.00 \pm 0.09, AL-CR = 1.09 \pm 0.18, KO-AL = 0.63 \pm 0.15, and KO-CR = 0.91 \pm 0.18). There was no significant main effect of diet ($P = 0.262$), but there

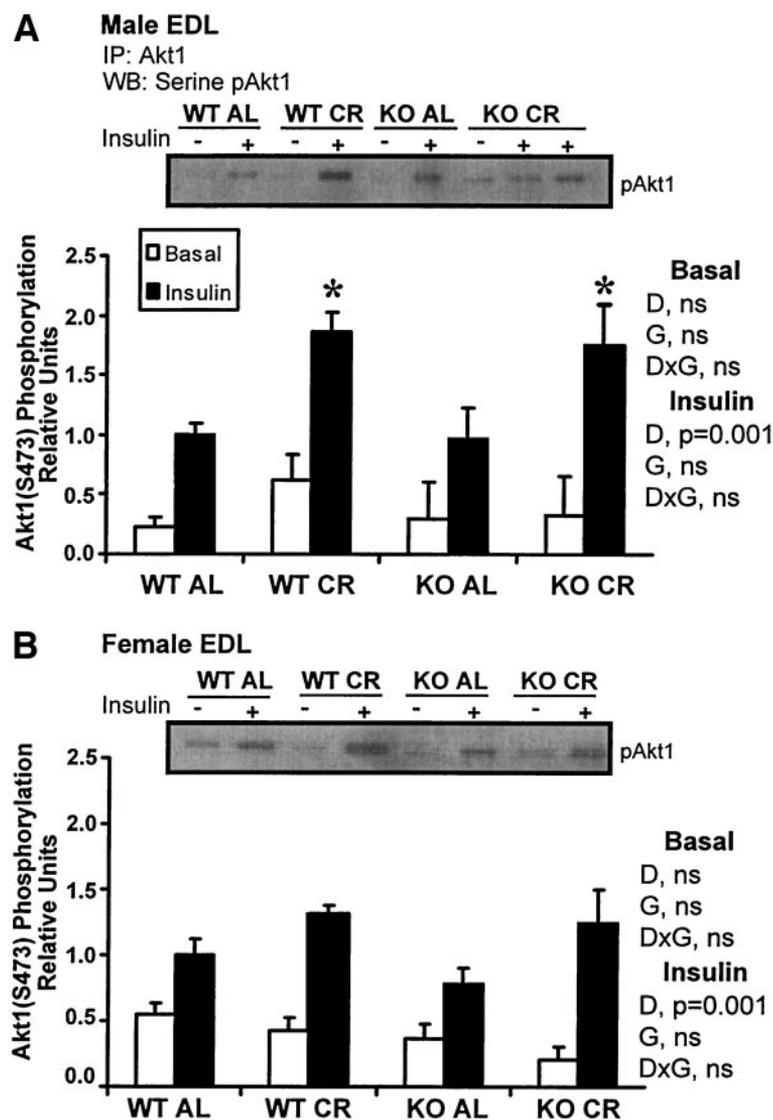


FIG. 3. Akt1(Ser⁴⁷³) phosphorylation in EDL. Immunoprecipitated Akt1 from EDL without (□) or with (■) insulin, analyzed by immunoblot with anti-phosphoserine Akt. Male (A) or female (B) data are means \pm SE (basal: $n = 3-4$; insulin: $n = 6-8$). Data within each sex and insulin level analyzed separately by two-way ANOVA. D, main effect of diet; G, main effect of genotype; D \times G, interaction between main effects. Tukey post hoc analysis was performed for a significant main effect. * $P < 0.05$ CR vs. AL.

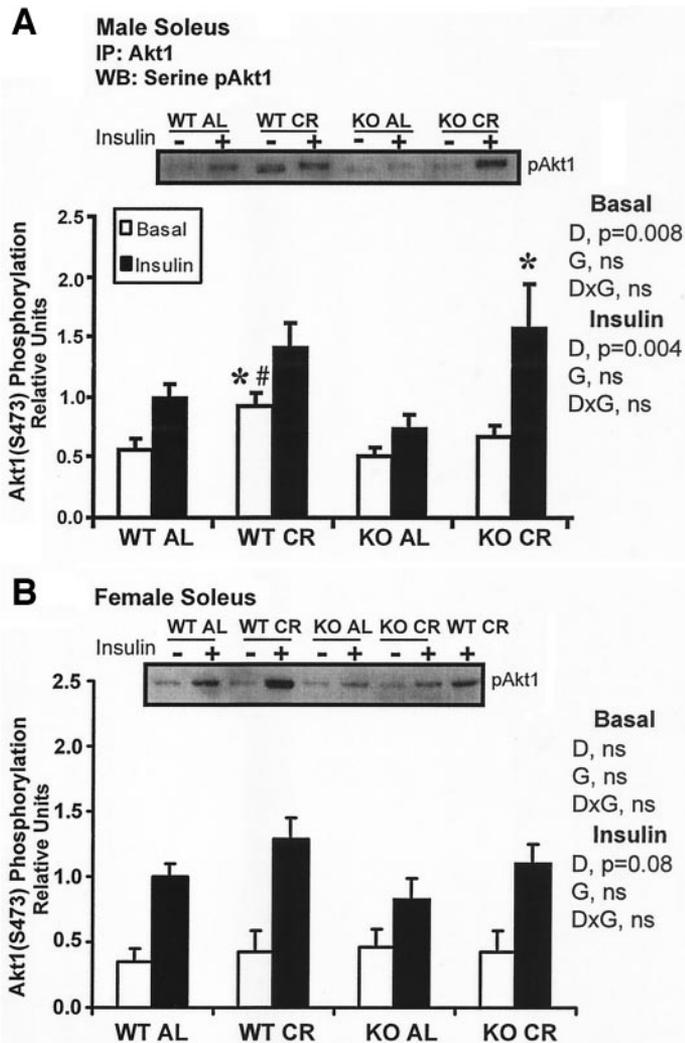


FIG. 4. Akt1(Ser⁴⁷³) phosphorylation in soleus. Immunoprecipitated Akt1 from male (A) or female (B) soleus without (□) or with (■) insulin, analyzed by immunoblot with anti-phosphoserine Akt. Data are means \pm SE (basal: $n = 3-7$; insulin: $n = 6-8$). Data within each sex and insulin level analyzed separately by two-way ANOVA. D, main effect of diet; G, main effect of genotype; D \times G, interaction between main effects. Tukey post hoc analysis was performed for a significant main effect. * $P < 0.05$ CR vs. AL.

was a nonsignificant trend for a main effect of genotype ($P = 0.096$).

DISCUSSION

The major new findings of this study are that 1) Akt2 was essential for the full effect of brief calorie restriction compared with ad libitum to increase insulin-stimulated 2DG uptake at a physiological insulin concentration, 2) increased Akt1 phosphorylation for insulin-treated calorie-restricted muscles compared with ad libitum controls was insufficient to replace Akt2 for insulin-stimulated glucose uptake, and 3) an Akt2-independent mechanism accounted for a significant increase in insulin-stimulated 2DG with calorie restriction versus ad libitum only in the soleus of male KO mice.

Recently, we found that 20 days of calorie restriction compared with ad libitum feeding increased insulin-stimulated Akt2 phosphorylation and not Akt1 phosphorylation above ad libitum levels in rat epitrochlearis incubated

with insulin (23). These results suggested that Akt2 plays an important role in mediating the calorie restriction effects on insulin-stimulated glucose uptake. Using a mouse null for Akt2, we now provide compelling evidence supporting this interpretation.

Akt2-null mice have impaired glucose tolerance and a defect in insulin-stimulated glucose uptake in isolated muscle (18,19). Our results are also consistent with whole-body and skeletal muscle insulin resistance for KO compared with WT mice. Serum insulin concentration was higher in KO compared with WT mice of both sexes. In both the EDL and soleus of KO-AL mice, a physiologic insulin concentration was insufficient to significantly enhance glucose uptake above basal.

Akt2 was essential for the full effect of calorie restriction on 2DG uptake in insulin-stimulated skeletal muscle. In all WT muscles studied, insulin-stimulated glucose uptake was increased for calorie restriction compared with ad libitum feeding. In contrast, for KO animals, insulin-stimulated 2DG uptake was not significantly increased in calorie restriction compared with ad libitum in EDL of either sex or female soleus. Only in the soleus of male KO mice did calorie restriction cause a significant increase of insulin-stimulated 2DG through an Akt2-independent pathway. However, insulin-stimulated 2DG uptake in male WT-CR soleus was still substantially higher (~ 1.6 -fold) than male KO-CR soleus. Without exception, insulin-stimulated 2DG uptake was significantly greater in muscles from WT-CR mice compared with respective sex-matched muscles from KO-CR mice at this physiologic insulin concentration.

The calorie restriction-induced increase in insulin-stimulated 2DG uptake in WT muscle corresponded with the consistent calorie restriction effect on Akt2 serine phosphorylation in insulin-stimulated muscle. In WT mice, Akt2 phosphorylation in insulin-stimulated muscle was approximately two- to threefold greater for calorie restriction versus ad libitum regardless of sex. Previously in male rat epitrochlearis, we found an approximately twofold increase in insulin-stimulated Akt2 serine phosphorylation with calorie restriction versus ad libitum (23). These results demonstrate a robust calorie restriction enhancement of insulin-stimulated Akt2 phosphorylation in muscles from both species.

Akt1 serine phosphorylation in insulin-stimulated muscles was also increased by ~ 1.5 - to 2-fold for calorie restriction versus ad libitum in mouse EDL and soleus regardless of genotype or sex. At first glance, these results appear to conflict with our findings in rat skeletal muscle, in which there was no difference in insulin-stimulated Akt1 serine phosphorylation between ad libitum and calorie restriction. However, there was a significant $\sim 30\%$ decrease in Akt1 abundance in rat skeletal muscle (23), and when insulin-stimulated Akt1 phosphorylation was expressed relative to abundance (pAkt1/Akt1), there was an ~ 1.5 -fold increase in insulin-stimulated Akt1 phosphorylation for calorie restriction versus ad libitum. Unlike rat muscle, Akt1 abundance in mouse muscle was not reduced with calorie restriction. In the absence of reduced Akt1 abundance, we would predict phosphorylation in insulin-stimulated muscles to be higher for calorie restric-

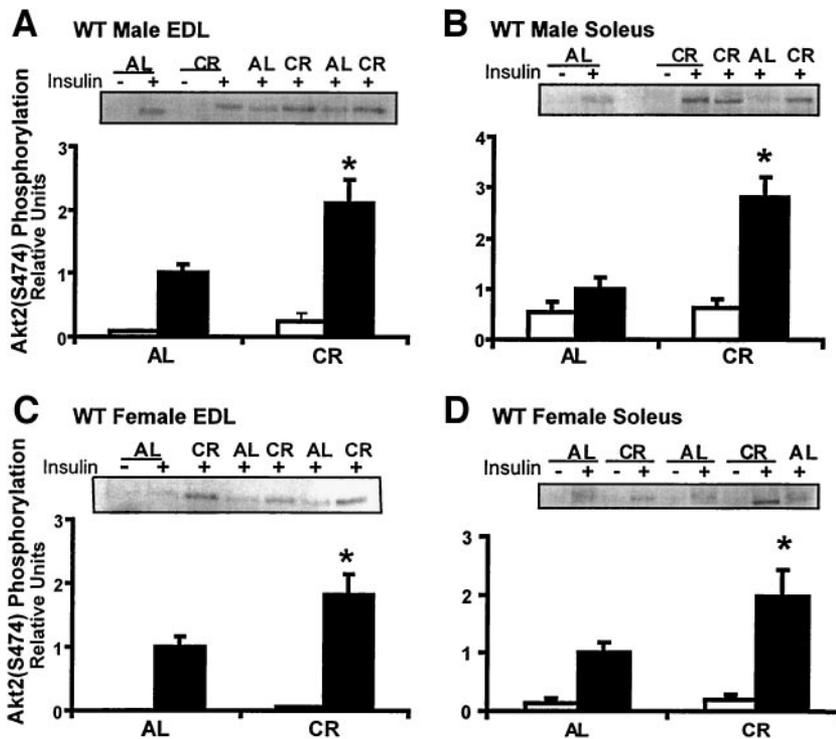


FIG. 5. Akt2(Ser⁴⁷⁴) phosphorylation in male EDL (A) and soleus (B) or female EDL (C) and soleus (D) from WT. Immunoprecipitated Akt2 from EDL or soleus incubated without (□) or with (■) insulin, analyzed by immunoblot with anti-phosphoserine Akt. Data are means \pm SE (basal: $n = 4-5$; insulin: $n = 8-10$). Data analyzed by Student's *t* test. * $P < 0.05$ CR vs. AL.

tion compared with ad libitum, consistent with the results now reported in mouse muscle.

Despite the 1.5- to 2-fold calorie restriction-induced increase in Akt1 serine phosphorylation, Akt1 was not sufficient to replace Akt2 for insulin-stimulated glucose uptake. A similar result occurred in primary adipocytes cultured from Akt2-null mice, where overexpression of Akt1 by approximately twofold was unable to eliminate defective insulin-stimulated glucose uptake (16), suggesting an isoform-specific function of Akt2 on insulin-stimulated glucose uptake that was not replaceable with elevated Akt1 function. Furthermore, calorie restriction-enhanced Akt1 serine phosphorylation did not correspond to insulin-stimulated 2DG uptake. In insulin-stimulated male EDL, calorie restriction increased Akt1 serine phosphorylation by approximately twofold above ad libitum in both WT and KO, but only in WT did calorie restriction cause improved insulin-stimulated glucose uptake. These results demonstrate an inability for elevated phosphorylation of Akt1 to replace Akt2 for insulin-stimulated glucose uptake in skeletal muscle of Akt2 null mice.

Calorie restriction also increased insulin-stimulated 2DG uptake in male KO soleus, indicating an Akt2-independent mechanism for this calorie restriction effect. Although 2DG uptake was lower for KO-CR compared with WT-CR, this result indicates that calorie restriction, at least in male soleus, can act through an alternative mechanism. It remains possible that, with sufficiently high insulin, calorie restriction might enhance insulin action in other muscles of both sexes. Nonetheless, calorie restriction is typically effective in the face of attenuated rather than elevated insulinemia, arguing against this mechanism as being physiologically relevant. Akt1 is a possible candidate for the Akt2-independent mechanism. In the insulin-stimulated soleus from male KO mice, both Akt1 serine phosphorylation and 2DG uptake were greater for the CR

compared with the AL group, raising the possibility of a relationship between these processes. However, among the other insulin-stimulated muscles from KO mice, there was not a consistent correspondence between calorie restriction effects on 2DG uptake and Akt1 serine phosphorylation, suggesting that with the possible exception of the soleus from male KO-CR mice, another mechanism is likely to be important. Atypical protein kinase C isoforms λ and ζ are attractive candidates for the Akt2-independent mechanism.

Calorie restriction also increased basal 2DG in mouse muscle, and this calorie restriction effect was unrelated to the presence of Akt2. Calorie restriction compared with ad libitum feeding significantly increased basal glucose uptake in EDL, regardless of genotype or sex, and in male WT soleus. There was no significant effect of calorie restriction on AMPK phosphorylation, arguing against this as a mechanism for the diet effect on basal 2DG uptake. Previously, we found elevated basal glucose uptake in calorie restriction compared with ad libitum feeding in mouse epitrochlearis with a trend for a similar increase in the EDL and soleus of CR versus AL mice of both sexes (4). In rat skeletal muscle, the effect of calorie restriction on glucose transport is specific to the insulin-mediated pathway without effect on basal glucose transport or basal cell surface GLUT4 content (6). It is possible that in basal mouse muscle, calorie restriction elevates cell surface GLUT4 and/or GLUT1.

Calorie restriction has been typically shown to reduce serum insulin, and in this study, calorie restriction reduced serum insulin in male WT and KO. Unexpectedly, calorie restriction led to increased serum insulin in female KO-CR. The reason for divergence in calorie restriction effects for male and female KO in serum insulin is not known, but there was no evidence that elevated insulin was a compensatory response for insulin resistance in skeletal mus-

cle of the female KO-CR compared with WT-CR mice. Insulin was measured in serum from fasted mice. It would be valuable to know the effects of calorie restriction on whole-body glucoregulation and insulin sensitivity in female KO mice in response to a glucose challenge.

In conclusion, without exception, the calorie restriction-induced increase above ad libitum feeding for insulin-stimulated 2DG uptake was greater in WT compared with KO. In three of four muscles studied from Akt2-deficient mice, insulin-stimulated 2DG uptake was not increased for CR compared with AL muscles. As in rat epitrochlearis (23), calorie restriction substantially enhanced Akt2 serine phosphorylation above ad libitum in insulin-stimulated WT mouse EDL and soleus. Akt1 phosphorylation was also increased in insulin-stimulated EDL and soleus with calorie restriction, regardless of genotype or sex, but increased Akt1 serine phosphorylation was insufficient to replace Akt2 for insulin-stimulated glucose uptake in Akt2-deficient muscles. The data support the hypothesis that Akt2 is crucial for the full effect of calorie restriction on elevating glucose uptake in skeletal muscle with a physiologic insulin concentration.

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