

# Calorie restriction increases muscle insulin action but not IRS-1-, IRS-2-, or phosphotyrosine-PI 3-kinase

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**Davidson, Robert T., Edward B. Arias, and Gregory D. Cartee.** Calorie restriction increases muscle insulin action but not IRS-1-, IRS-2-, or phosphotyrosine-PI 3-kinase. *Am J Physiol Endocrinol Metab* 282: E270–E276, 2002; 10.1152/ajpendo.00232.2001.—Skeletal muscle insulin sensitivity improves with a moderate reduction in caloric intake. We studied possible mechanisms in calorie-restricted [CR: 60% ad libitum (AL) intake] compared with AL rats, utilizing a time-matched feeding protocol (3, 5, 10, or 20 days). Visceral fat mass was lower for CR vs. AL at 10 and 20 days, but insulin-stimulated muscle 3-*O*-methylglucose transport was higher in CR vs. AL rats only at 20 days. Fructose 6-phosphate (precursor for the hexosamine biosynthetic pathway, which has inverse relationship with insulin sensitivity) was reduced only at 3 days of CR. Insulin stimulation of insulin receptor substrate (IRS)-1-, IRS-2-, and antiphosphotyrosine-associated phosphatidylinositol 3-kinase (PI3K) was similar for CR and AL. A PI3K inhibitor, wortmannin, reduced insulin-stimulated 3-*O*-methylglucose transport to basal levels, regardless of diet. With brief time-matched CR, reduced visceral fat mass precedes increased insulin sensitivity; transient reduction in fructose 6-phosphate may trigger more persistent changes but does not coincide with enhanced insulin action; and PI3K is essential for insulin-stimulated 3-*O*-methylglucose transport in CR as well as AL rats, although insulin-stimulated PI3K is not significantly greater in CR compared with AL animals.

glucose transport; food restriction; dietary restriction; insulin signaling

MODERATE CALORIE RESTRICTION (CR; consuming 60–75% of ad libitum intake) improves insulin action, and a pivotal adaptation to CR is the enhanced insulin-stimulated glucose transport in skeletal muscle (18). Skeletal muscle accounts for most of the whole body insulin-mediated glucose clearance (14, 19), and glucose transport appears to be the rate-limiting step of glucose metabolism in skeletal muscle (28). Little is known about the cellular mechanisms underlying this process. Elucidating these mechanisms has importance for both fundamental biology and clinical application. Our overall hypothesis is that CR induces changes in the skeletal muscle insulin-signaling pathway, secondary to changes in the metabolic milieu of

the muscle (for example, changes in concentrations of key metabolites).

In this study, our first goal was to evaluate the time course for CR effects on glucose transport. We previously characterized the time course for increased insulin sensitivity with brief CR and found increased glucose transport after 5 days of CR despite no significant reduction in visceral fat mass detected at 5 days of CR, suggesting that a substantial decline in this tissue mass was not essential for the improved insulin action (4). In that study, CR animals were provided their feed in a single daily allotment, whereas controls had continuous access to feed. With this conventional (once daily) feeding approach, CR rats eat all of their food within 2–3 h of being fed, whereas ad libitum-fed rats eat throughout the day. To evaluate the effects of reduced food intake, independent of temporal differences in consumption, we studied control and CR groups of rats that were matched for timing of feeding, and we found that CR elicited enhanced insulin action compared with time-matched controls (18). Because we previously compared time-matched groups after only 20 days of CR, we have now investigated the effects on glucose transport of a time-matched feeding protocol with shorter periods (3, 5, and 10 days) of CR to determine whether enhanced insulin action preceded a detectable decline in visceral fat mass under time-matched feeding conditions.

Brief (5–28 days) CR does not increase insulin receptor number, binding affinity, or tyrosine kinase activity (1, 7), suggesting a postreceptor mechanism. However, CR does not induce an increased abundance of insulin receptor substrate (IRS)-1 or IRS-2 (the two IRS proteins expressed by skeletal muscle) or p85 phosphatidylinositol 3-kinase (PI3K) abundance (13). There is also no significant increase in IRS-1-associated PI3K activity with CR (12, 13). Furthermore, insulin-stimulated glucose transport is also increased in transgenic mice that lack IRS-1, implicating an IRS-1-independent mechanism (16). None of these studies used a time-matched feeding protocol.

Considerable evidence indicates that insulin stimulation of glucose transport is dependent on PI3K activity (8, 24); therefore, our second goal was to assess the

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possible influence of CR on PI3K and the role of PI3K in CR-induced increase in insulin-stimulated glucose transport. We used four approaches to assess the relationship between CR and PI3K in muscle. First, we assessed IRS-1-PI3K activity in time-matched CR rats. Second, we determined the effect of CR on IRS-2-PI3K activity, which had not been previously evaluated with CR. Third, we determined the influence of CR on phosphotyrosine (pTyr)-associated PI3K activity in muscle. The rationale for this experiment was that another intervention, a single bout of exercise that increases insulin-stimulated glucose transport without increasing IRS-1-associated PI3K (10, 25, 26), has been shown to elevate pTyr-associated PI3K (27). It seemed possible that CR might also amplify this aspect of insulin signaling. Finally, we used wortmannin, a specific PI3K inhibitor, to examine whether the CR-induced increase in insulin-stimulated glucose transport requires the insulin-mediated increase in PI3K activity.

Our third goal was to determine the influence of brief CR on skeletal muscle concentration of fructose 6-phosphate (F-6-P). F-6-P is a substrate for glutamine:fructose-6-phosphate amidotransferase (GFAT), the first and rate-limiting enzyme of the hexosamine biosynthetic pathway. High flux through this pathway is associated with insulin resistance (21); therefore, we reasoned that reduced flux through this pathway may be associated with increased insulin sensitivity with CR. Consistent with this idea, we recently found that 20 days of CR led to a reduction in muscle concentration of UDP-N-acetylhexosamine (formed by the hexosamine pathway) (18). We now test the hypothesis that muscle F-6-P concentration declines with CR, which, in turn, could potentially account for lower UDP-N-acetylhexosamine levels. We also measured muscle concentration of glucose 6-phosphate (G-6-P), the immediate precursor for F-6-P, which is an important allosteric regulator of glycogen metabolism.

## METHODS

**Animal care and feeding.** Animal procedures were approved by the University of Wisconsin-Madison Research Animal Review Committee. For both *experiment 1* (3, 5, and 10 days of CR) and *experiment 2* (20 days of CR), male Fischer 344 × Brown Norway, F1 generation rats (F344BNF1/Nia), aged 4 mo, were obtained through the National Institute on Aging from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed individually in wire-bottomed cages in a controlled environment (22–25°C, 40–50% humidity, 12:12-h light-dark cycle with lights on 0600–1800). Rats were given ad libitum access to water and feed (Teklad Rodent Diet no. 8604, Harlan Teklad, Madison, WI) for a 1-wk acclimation period.

After acclimation, rats in *experiment 1* were randomly assigned to treatment groups. Baseline feed intake was assessed over 7 days for each rat by presenting feed ad libitum and measuring feed eaten, remaining, and spilled. All rats were familiarized to a meal-feeding protocol for 7 days, wherein they were given a portion of 100% of their individual baseline feed allotment in three 1.5-h periods (30% at 0600, 30% at 1500, and 40% at 2000). After familiarization with the meal-feeding protocol, the ad libitum (AL) group continued receiving 100% of baseline intake for 10 (10AL) days, and the

calorie-restricted (CR) groups received 60% of their individual baseline intake according to the same meal-feeding protocol for 3 (3CR), 5 (5CR), or 10 (10CR) days. Feed intake and loss by spillage were measured daily for all rats.

Rats in *experiment 2* were ranked by weight and assigned to control or treatment groups such that initial weight means ± SE were similar for all groups. Baseline feed intake assessment was as in *experiment 1*. The meal-feeding protocol familiarization was as in *experiment 1* except that meal proportions were slightly adjusted to ensure complete consumption of feed (35% at 0600, 25% at 1500, and 40% at 2000). After familiarization with the meal-feeding protocol, the AL group continued to receive 100% of baseline intake for 20 (20AL) days while the CR group received 60% of their individual baseline intake for 20 (20CR) days. Feed intake and spillage were measured daily for all rats. As necessary to correct for spillage, adjustments in feed allotments were made to ensure that AL and CR animals consumed ~100 and 60%, respectively, of their individual baseline intake.

Animals received their 0600 meal on the day they were killed, and at 1000 they were moved from the animal facility to the laboratory and housed individually with water ad libitum. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip) between 1300 and 1600. Order of anesthetization was alternated among groups. Epitrochlearis muscles were dissected out for in vitro incubations. A plantaris muscle was also dissected from each rat in *experiment 1* and immediately freeze-clamped and stored at –80°C until analysis. The epididymal fat pads and retroperitoneal fat pads were removed and weighed.

**3-O-Methylglucose transport.** Glucose transport activity was determined in isolated epitrochlearis muscle, as previously reported (18). Briefly, dissected epitrochlearis muscles were incubated (30°C) with continuous shaking in flasks of oxygenated (continuously gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>) Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 2 mM sodium pyruvate, and 6 mM mannitol in the absence or presence of insulin (500 μU/ml), with or without the addition of 0.5 μM wortmannin (as noted), and with continuous gassing (95% O<sub>2</sub>-5% CO<sub>2</sub>) and shaking. One epitrochlearis muscle from each rat was used for assessing basal glucose transport, and the contralateral muscle was incubated with insulin. In a subset of *experiment 2*, one epitrochlearis muscle from each rat was incubated in the absence or presence of insulin (500 μU/ml), with the contralateral epitrochlearis muscle additionally receiving wortmannin (0.5 μM). After 30 min of incubation, muscles were transferred to flasks of KHB-BSA containing 8 mM 3-O-[<sup>3</sup>H]methylglucose (3MG; 0.25 mCi/mmol; NEN Life Science Products, Boston, MA), 2 mM [<sup>14</sup>C]mannitol (0.1 mCi/mmol; Amersham Pharmacia, Piscataway, NJ), and insulin and wortmannin levels as in the first incubation. After 15 min, muscles were blotted, trimmed, freeze-clamped, and stored at –80°C until analyzed. Rate of 3MG transport was determined as previously described (2) by use of a portion of the muscle after homogenization in 0.3 N perchloric acid.

**Glycogen concentration.** Muscle glycogen was determined in PCA homogenates of plantaris (*experiment 1*) and epitrochlearis (*experiment 2*) by the fluorometric amyloglucosidase method (22).

**G-6-P and F-6-P concentrations.** G-6-P and F-6-P in plantaris muscles were measured by fluorometric procedure, as previously described (20).

**Immunoprecipitation.** A portion of the epitrochlearis muscle of animals in *experiment 2* was used for insulin signaling assessment by measuring PI3K activity, as previously described (13). Frozen epitrochlearis muscle was homogenized

in ice-cold homogenization buffer [50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate (NaPP), 2 mM  $\text{Na}_3\text{VO}_4$ , 10 mM NaF, 2 mM EDTA, 2 nM phenylmethylsulfonyl fluoride (PMSF), 5  $\mu\text{g}/\text{ml}$  leupeptin, 1% NP-40, and 10% glycerol]. A portion of the resultant supernatant was used for determination of protein concentration using bicinchoninic acid. Immunoprecipitation of IRS-1, IRS-2, and pTyr-PI3K was performed on epitrochlearis homogenate by use of polyclonal anti-IRS-1 antibody, raised against the carboxy-terminal 14-amino acid peptide [(C)YASINFQKQPEDRQ] of rat liver IRS-1; polyclonal anti-IRS-2 antibody, raised against a GST fusion protein containing amino acids 976–1094 of mouse IRS-2 from Upstate Biotechnology (Lake Placid, NY), cat. nos. 06–248 and 06–506, respectively; or monoclonal anti-pTyr clone PT-66 antibody, derived from the hybridoma produced by the fusion of mouse myeloma cells and splenocytes from a mouse immunized with a phosphotyrosine-BSA conjugate; Sigma (St. Louis, MO), cat. no. P3300.

**IRS- and pTyr-associated PI3K assays.** IRS-1, IRS-2, and pTyr-associated PI3K activities were assessed as previously described (13). The reaction mixture contained 30  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP (NEN Life Science Products, Boston, MA) and 20  $\mu\text{g}$  of phosphatidylinositol (AVANTI Polar Lipids, Alabaster, AL). Results are reported as counts per minute per milligram protein per 20 min.

**Statistical analysis.** Differences among diet groups were analyzed by one-way ANOVA with a Tukey-Kramer multiple comparisons post hoc test for multiple group statistics, or by Student's *t*-test when only two groups were compared (In-Stat, GraphPad Software, San Diego, CA). A *P* value < 0.05 was considered to be significant.

## RESULTS

**Food intake.** Figure 1 shows average food intake for rats for *experiment 2*. A similar pattern of eating occurred for *experiment 1* (which ended after 10 days of

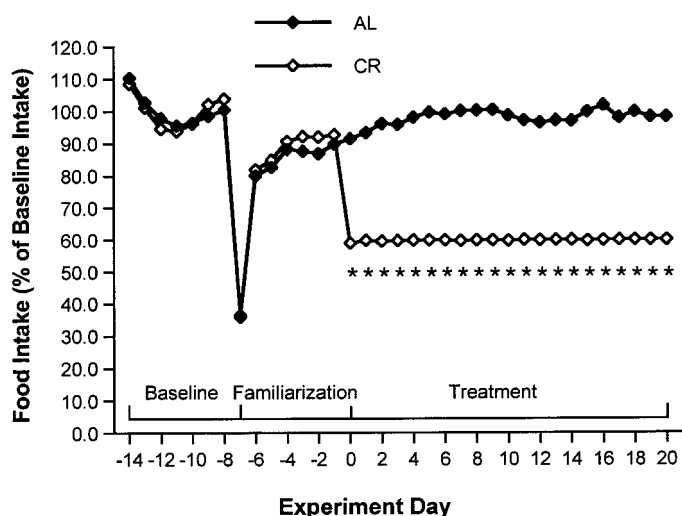


Fig. 1. Feed intake (as a percentage of baseline intake) of rats during ad libitum baseline intake determination (days -14 to -8), meal-feeding familiarization (days -7 to 0), and 20 days of meal feeding of 100% (AL) or 60% (CR) of baseline ad libitum intake (days 1–20). Data are from *experiment 2* and represent group mean intake ( $n = 40$  rats/group). Similar feed intake patterns were observed for *experiment 1*, with groups terminating at days 3, 5, and 10 of treatment. Feed intake was lower for CR groups compared with the AL group for each day of the treatment period (days 1–20,  $*P < 0.0001$ ) by Student's *t*-test.

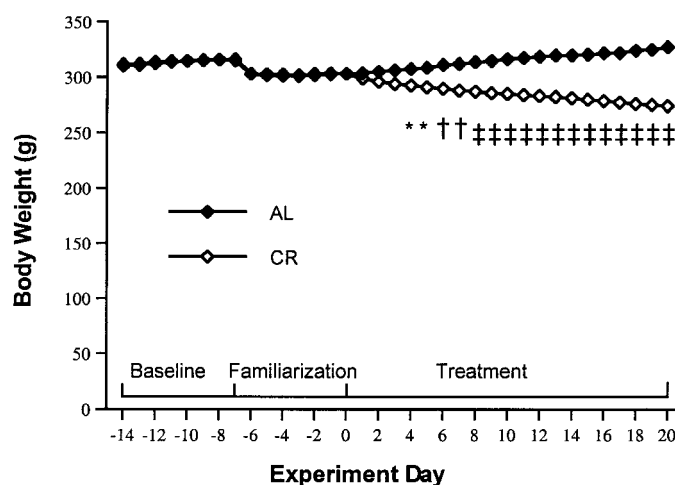


Fig. 2. Body weight of rats during ad libitum baseline intake determination (days -14 to -8), meal feeding familiarization (days -7 to 0), and 20 days of meal feeding of 100% (AL) or 60% (CR) of baseline ad libitum intake (days 1–20). Data are from *experiment 2* and represent group mean weight ( $n = 40$  rats/group). Similar patterns in body weight were observed for *experiment 1*, with groups terminating at days 3, 5, and 10 of treatment. AL body weight different from CR body weight on the following days: days 4–5  $*P < 0.05$ , days 6–7  $\dagger P < 0.001$ , days 8–20  $\ddagger P < 0.0001$ , by Student's *t*-test.

CR). On the first day of the meal familiarization period (designated as “day -7” on Fig. 1), feed intake was reduced compared with baseline values. However, rats rapidly adjusted their feeding behavior so that during the 10-day treatment period for *experiment 1*, mean feed intake of the 10AL group equaled 97% of their baseline intake, and during the 20-day treatment period of *experiment 2*, mean feed intake of the 20AL group equaled 98% of their baseline intake. The mean daily feed intake of CR groups in *experiment 1* (3CR, 5CR, and 10CR) equaled 65–67% of the 10AL group. The feed intake of the 20CR group during the treatment period equaled 61% of the 20AL group.

**Body and fat pad weights.** In each experiment there was no difference in initial animal weights; however, as expected, final body weight was lower in CR compared with AL groups (Fig. 2,  $P < 0.0001$ ). Retroperitoneal fat pad mass was significantly lower in the 5CR, 10CR, and 20CR groups than in respective AL groups in *experiments 1* and 2 (Table 1,  $P < 0.05$ ). Epididymal fat pad mass did not significantly differ among treatment groups in *experiment 1* but was significantly lower in 20CR compared with 20AL in *experiment 2* ( $P < 0.0001$ ).

**Muscle glycogen concentration.** Muscle glycogen concentration did not differ among treatment groups in either experiment (data not shown).

**G-6-P and F-6-P concentrations.** Muscle G-6-P concentration was not significantly different among diet groups (Fig. 3A). Muscle F-6-P concentration (Fig. 3B) was significantly lower in the 3CR compared with the 10AL group ( $P < 0.05$ ). Muscle F-6-P concentrations were not different in the 20CR compared with the 20AL group.

**Epitrochlearis 3MG transport.** There was no significant effect of 3, 5, or 10 days of CR on either basal or

Table 1. *Body and fat pad weight*

Treatment	Initial Body Wt, g	Final Body Wt, g	Fat Pad Wt, g	
			Epididymal	Retroperitoneal
<i>Experiment 1</i>				
10AL	321.9 ± 5.7	319.3 ± 4.1 <sup>a</sup>	4.21 ± 0.17 <sup>b</sup>	2.74 ± 0.12 <sup>c</sup>
3CR	340.3 ± 6.5	319.1 ± 6.0	4.00 ± 0.18	2.42 ± 0.14
5CR	344.7 ± 6.5	321.2 ± 6.6	3.97 ± 0.29	2.04 ± 0.16 <sup>d</sup>
10CR	330.3 ± 6.9	297.9 ± 5.5 <sup>d</sup>	3.61 ± 0.21	2.00 ± 0.18 <sup>d</sup>
<i>Experiment 2</i>				
20AL	310.3 ± 4.9	327.3 ± 4.7	4.03 ± 0.21	2.82 ± 0.12
20CR	311.6 ± 4.8	274.0 ± 4.1 <sup>e</sup>	2.14 ± 0.18 <sup>e</sup>	1.23 ± 0.09 <sup>e</sup>

Values are means ± SE; *experiment 1*:  $n = 12/\text{group}$ ; *experiment 2*:  $n = 40/\text{group}$  except epididymal fat pad ( $n = 16/\text{group}$ ) and retroperitoneal fat pad ( $n = 24/\text{group}$ ). Feeding protocols consisted of 10 days of ad libitum feeding (10AL) or of 3, 5, or 10 days of calorie restriction (3CR, 5CR, and 10CR, respectively) in *experiment 1*, and 20 days of AL or CR in *experiment 2*. ANOVA for *experiment 1*: final body wt, <sup>a</sup> $P = 0.02$ ; epididymal wt, <sup>b</sup> $P = 0.003$ ; retroperitoneal wt, <sup>c</sup> $P = 0.003$ . Significantly different from respective AL group mean value by Tukey-Kramer multiple comparison test (<sup>d</sup> $P < 0.05$ ) or Student's *t*-test (<sup>e</sup> $P < 0.0001$ ).

insulin-stimulated 3MG transport in epitrochlearis (data not shown). As expected, basal 3MG transport did not differ between 20AL and 20CR groups (Fig. 4). Insulin-stimulated 3MG transport was significantly greater in the 20CR compared with the 20AL group ( $P = 0.006$ ). Wortmannin inhibited 3MG transport in insulin-stimulated muscles to values not different from basal levels (data not shown).

**PI3K activity.** IRS-1, IRS-2, and pTyr-associated PI3K activities were determined from 20AL and 20CR rat epitrochlearis muscle (Fig. 5). There was no significant difference between AL and CR groups for IRS-1-, IRS-2-, or pTyr-associated PI3K activity under basal or insulin-stimulated conditions. These values were also not significantly different between diet groups when expressed as an increase above basal level (insulin-treated value – basal value) or as a degree of increase (insulin-treated value ÷ basal value). Wortmannin treatment reduced pTyr-PI3K activity in insulin-treated muscles to values not different from basal levels (data not shown).

## DISCUSSION

In this study, we have matched the timing of eating in the CR and control groups because timing of eating can influence metabolism and gene expression (15, 22), and failure to control for this variable may confound the identification of changes that are related to amount, rather than timing, of food intake. The major aims of the experiments reported here were to 1) assess effects of a time-matched feeding protocol on insulin-stimulated 3MG glucose transport in skeletal muscle after 3, 5, 10, and 20 days of CR; 2) investigate effects of time-matched CR on IRS-1, IRS-2-, and pTyr-associated PI3K activity and to determine whether the CR-induced increase in insulin sensitivity is wortmannin inhibitable; and 3) assess effects of brief CR on

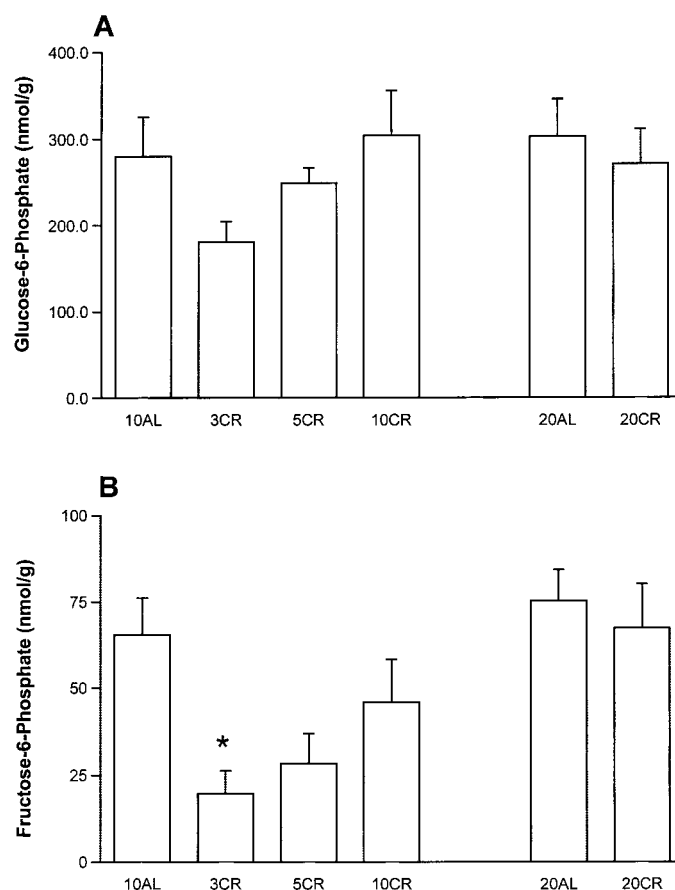


Fig. 3. Glucose 6-phosphate (G-6-P, A) and fructose 6-phosphate (F-6-P, B) concentrations in plantaris muscle. Data are means ± SE from *experiment 1* (10AL, 3CR, 5CR, and 10CR;  $n = 12/\text{group}$ ) and *experiment 2* (20AL and 20CR;  $n = 12/\text{group}$ ). \* $P < 0.05$ , different from 10AL by Tukey-Kramer multiple comparisons post hoc ANOVA.

F-6-P (initial substrate for the hexosamine biosynthetic pathway) in skeletal muscle.

We previously reported an increase in insulin-stimulated 3MG transport in skeletal muscle of rats (8

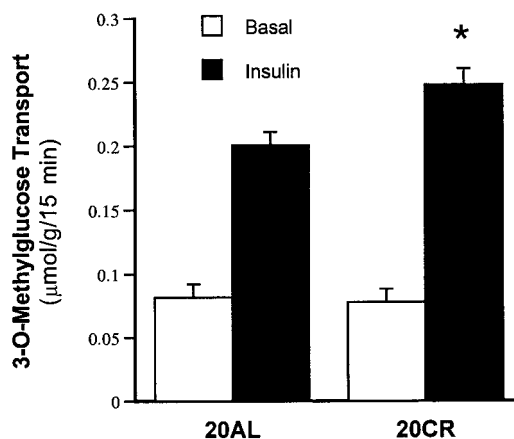


Fig. 4. Rate of 3-O-methylglucose (3MG) transport in epitrochlearis muscle from 20AL and 20CR rat groups. 3MG transport was determined after incubation without insulin (Basal; open bars) or with 500  $\mu\text{U}/\text{ml}$  insulin (Insulin, filled bars). Data are means ± SE for  $n = 40$  rats/group. \* $P = 0.006$ , 20CR significantly different from 20AL for insulin-stimulated muscles by Student's *t*-test.

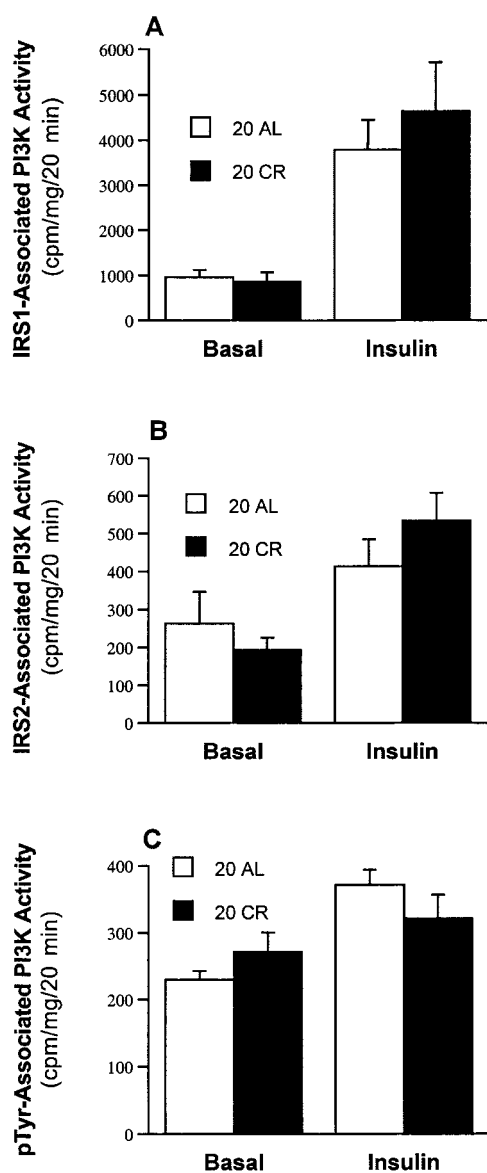


Fig. 5. Phosphatidylinositol 3-kinase (PI3K) activity in immunoprecipitated samples prepared using epitrochlearis muscle from rats in 20AL and 20CR groups without insulin (Basal; open bars) or with 500  $\mu$ U/ml insulin (Insulin; filled bars). Data are means  $\pm$  SE. There are no significant differences between AL and CR groups. A: insulin receptor substrate (IRS)-1-associated PI3K activity for  $n = 8$ /group; B: IRS-2-associated PI3K activity for  $n = 8$ /group; C: pTyr-associated PI3K activity for Basal ( $n = 7$ ) and Insulin ( $n = 14$ ).

mo-old, female, F344) after 5 or 20 days of once daily feeding CR (4). Our current results, using a time-matched feeding CR protocol, demonstrate a significant increase in insulin-stimulated 3MG transport after 20 days of CR but not after 3, 5, or 10 days of CR. In our earlier study, the CR rats consumed their allotment of feed within 3 h, whereas the AL-fed controls ate throughout the day. Thus, on the day the animals were killed, the CR animals had been without feed considerably longer, which may have influenced insulin sensitivity. We recently compared 20 days of once daily and time-matched feeding and found a significant increase in insulin-stimulated 3MG transport with

each CR group compared with AL-fed animals (18). Furthermore, there was no significant difference in glucose transport between the two CR feeding protocols. Taken together, these studies suggest that the increase in insulin-stimulated glucose transport that we previously observed after only 5 days of once daily feeding CR was likely due to differences in timing of eating. It is also possible that differences in the time courses between the current study and the original study with once daily feeding CR (4) were influenced by differences in age, gender, or rat strains. This earlier study also reported that the increase in insulin sensitivity preceded any change in abdominal fat mass changes, whereas our current study identified significant decreases in retroperitoneal fat mass before a significant increase in insulin-stimulated 3MG transport.

We found that CR did not significantly increase IRS-1-associated PI3K activity, confirming our previous results (12, 13). In each of these studies, there has been a nonsignificant trend for a slightly higher IRS-1-associated PI3K in the CR groups compared with the AL control group. We also extend our previous findings by demonstrating that brief CR does not alter IRS-2- or pTyr-associated PI3K activity. Furthermore, we found that wortmannin, a specific PI3K inhibitor, blocked the CR-induced increase in glucose transport. Taken together, these results suggest that PI3K activity is essential for improved insulin action with CR but that the insulin-stimulated increase in IRS-1/2-associated PI3K is not markedly elevated for CR compared with AL groups. Several possible mechanisms might account for these results. For example, it is conceivable that CR 1) alters cellular localization of signaling components; 2) acts on a post-PI3K signaling event; and/or 3) affects one or more components of a recently proposed pathway (involving the proteins Cbl, CAP, CrkII, C3G, and TC10), which is parallel to and acts in concert with the PI3K pathway (9, 23). Regardless of the putative effects on insulin signaling, the enhanced insulin-stimulated glucose transport can ultimately be accounted for by a greater translocation of the GLUT-4 glucose transporter to the cell surface of muscle from CR compared with AL rats (11).

After a single bout of exercise, insulin-stimulated glucose transport is increased in skeletal muscle (3, 5, 6). A recent study reported that insulin-stimulated pTyr-associated PI3K activity was higher in exercised compared with nonexercised muscle (27). Therefore, we considered the possibility that CR might have a similar effect, and we found that brief CR did not increase insulin-stimulated pTyr-associated PI3K activity, suggesting a difference in the mechanisms by which CR and exercise increase insulin sensitivity. Consistent with this interpretation, prior exercise leads to enhanced activation of glucose transport by insulin-independent stimuli (in vitro hypoxia) (5), but CR does not enhance hypoxia-stimulated glucose transport (13).

High flux through the hexosamine biosynthetic pathway is associated with insulin resistance (21), and we

previously found that muscle concentration of UDP-*N*-acetylhexosamine (formed in the hexosamine biosynthetic pathway) was lower after 20 days of CR compared with ad libitum controls (with time-matched feeding protocol) despite no change in GFAT activity (17). In light of these results, an attractive hypothesis was that CR would result in lower F-6-P values compared with time-matched controls, which might, in turn, account for the lower UDP-*N*-acetylhexosamine levels with CR. Our present findings identified a transient decrease in F-6-P at 3 days of CR treatment, but this decrement did not coincide with the lower levels of hexosamine metabolites that we previously found at 20 days of CR. The absence of a CR effect on GFAT activity was determined at a saturating F-6-P concentration (18). Perhaps CR caused a decreased Michaelis-Menten kinetic constant of GFAT for F-6-P, which could lead to lower accumulation of hexosamine metabolites.

In conclusion, the present results indicated that a moderate loss of visceral fat mass with CR preceded increased insulin-stimulated glucose transport when timing of food intake was matched with controls. Therefore, it remains possible that there is a link between the brief CR-induced changes in body composition and muscle insulin action. There was a decline in F-6-P at 3 days of CR that was reversed by 20 days of CR. The transient decline in F-6-P may trigger more persistent changes, but lower F-6-P concentration does not directly account for enhanced glucose transport with 20 days of CR. Insulin stimulation of glucose transport was inhibited by wortmannin in CR as well as AL rats, demonstrating an essential role for PI3K in activating glucose transport, regardless of diet. In addition, PI3K activity was not significantly different between AL and CR groups, suggesting that improved insulin sensitivity with brief CR is the result of enhanced signaling at a post-PI3K step, altered subcellular distribution of PI3K, and/or modification of PI3K-independent insulin signaling.

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