Calorie restriction increases cell surface GLUT-4 in insulin-stimulated skeletal muscle

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Dean, David J., Joseph T. Brozinick, J. R., Samuel W. Cushman, and Gregory D. Cartee. Calorie restriction increases cell surface GLUT-4 in insulin-stimulated skeletal muscle. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E957–E964, 1998.—Reduced calorie intake [calorie restriction (CR); 60% of ad libitum (AL)] leads to enhanced glucose transport without altering total GLUT-4 glucose transporter abundance in skeletal muscle. Therefore, we tested the hypothesis that CR (20 days) alters the subcellular distribution of GLUT-4. Cell surface GLUT-4 content was higher in insulin-stimulated epitrochlearis muscles from CR vs. AL rats. The magnitude of this increase was similar to the CR-induced increase in glucose transport, and GLUT-4 activity (glucose transport rate divided by cell surface GLUT-4) was unaffected by diet. The CR effect was specific to the insulin-mediated pathway, as evidenced by the observations that basal glucose transport and cell surface GLUT-4 content, as well as hypoxia-stimulated glucose transport, were unchanged by diet. CR did not alter insulin’s stimulation of insulin receptor substrate (IRS)-1-associated phosphatidylinositol 3-kinase (PI3K) activity. Muscle abundance of IRS-2 and p85 subunit of PI3K were unaltered by diet, but IRS-1 content was lower in CR vs. AL. These data demonstrate that, despite IRS-1-PI3K activity similar to AL, CR specifically increases insulin’s activation of glucose transport by enhancing the steady-state proportion of GLUT-4 residing on the cell surface.

food restriction; insulin signaling

Understanding the adaptive responses of organisms to the ebb and flow of energy availability has fundamental physiological importance. A cluster of pathophysiological outcomes (including insulin resistance, obesity, and type 2 diabetes mellitus) is intimately related to a chronic, positive energy balance, and many studies have focused on conditions of caloric overabundance. Previous research has also revealed that an energy deficit has an opposite effect on many of the same metabolic processes: e.g., moderate calorie restriction [CR; consuming ~60–75% of ad libitum (AL) intake] improves insulin action in various species including rats, rhesus monkeys, and humans (7, 18, 25, 28, 40).

Skeletal muscle accounts for most of the whole body insulin-mediated glucose clearance (16, 26), and glucose transport appears to be the rate-limiting step of glucose metabolism in skeletal muscle (45). Therefore, a pivotal adaptation to CR is the enhanced insulin-stimulated glucose transport in skeletal muscle (10, 15). This adaptation can occur rather rapidly, within 5–20 days of CR (7, 15), but very little is known about the cellular and molecular mechanisms that underlie this functional change. Therefore, the long-range goal of our research is to elucidate the mechanisms underlying the CR-induced increase in the insulin-mediated glucose transport by skeletal muscle.

GLUT-4, the predominant glucose transporter expressed by skeletal muscle, appears to account for most of the insulin-stimulated glucose transport in muscle. A close relationship between muscle GLUT-4 content and capacity for insulin-stimulated glucose transport has been observed (21), but we have found that CR does not alter muscle total GLUT-4 content (10). Under AL-fed conditions, the insulin-stimulated increase in glucose transport rate appears to be accounted for by an increase in the abundance of GLUT-4 residing on the cell surface (39). A reasonable hypothesis was that CR enhances insulin-stimulated glucose transport by increasing the translocation of GLUT-4 to the cell surface. Therefore, we evaluated the effect of CR on cell surface GLUT-4 content by exofacial photolabeling with 2-N-4-(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis(α-mannos-4-lyoxy)-2-propylamine (ATB-BMPA) in basal and insulin-stimulated muscles.

We also evaluated several other aspects of the CR-induced increase in glucose transport. In skeletal muscle, contractile activity and in vitro hypoxia increase glucose transport via a pathway distinct from that activated by insulin. The maximal effect of insulin on glucose transport is additive with contractile activity (13) or hypoxia (8), and wortmannin [a specific phosphatidylinositol 3-kinase (PI3K) inhibitor] entirely prevents insulin- but not contraction- or hypoxia-stimulated glucose transport (30, 43). As with insulin, contractile activity and hypoxia increase glucose transport by a mechanism involving increased appearance of GLUT-4 on the cell surface (8, 17). Thus it was possible that CR acted by a nonspecific mechanism, such as altering the susceptibility of the GLUT-4 translocation “machinery” to both insulin-dependent and insulin-independent signaling. Accordingly, we evaluated the influence of CR on glucose transport activated by in vitro hypoxia, which stimulates glucose transport by an insulin-independent pathway.

Insulin receptor substrate (IRS)-1 is an important substrate for the insulin receptor kinase (38). The
tyrosine-phosphorylated IRS-1 engages PI3K (38), and PI3K activity is essential for insulin-stimulated, but not hypoxia-stimulated, glucose transport in skeletal muscle (43). Therefore, to evaluate the effect of CR on a key aspect of the insulin signaling pathway, we also measured the PI3K activity associated with IRS-1 and the abundance of three important insulin signaling proteins in skeletal muscle: IRS-1, IRS-2, and the p85 subunit of PI3K.

Our results indicate that brief CR caused a specific increase in insulin-stimulated glucose transport rate without any effect on hypoxia-stimulated glucose transport. The increased glucose transport was largely attributable to more cell surface GLUT-4 in the insulin-stimulated muscles of CR compared with AL rats, but it was independent of a significant diet effect on glycogen concentration or IRS-1-associated PI3K activity in epistrochlearis muscles.

MATERIALS AND METHODS

Treatment of animals. Male Fischer 344 (F344) rats (Harlan Sprague Dawley, Indianapolis, IN) were housed singly on a 12:12-h light- (0600–1800) dark (1800–0600) cycle. After the animals had been in the Madison facility for 5–7 days, daily food consumption (NIH-31, PMI Feeds, St. Louis, MO) was monitored for 5–7 days to establish individual baseline food intake. Food consumption was determined by weighing the food provided and correcting for the amount of food not eaten, including spillage. Animals were randomly assigned to either AL or CR groups. CR animals received an allotment of food (NIH-31 supplemented with sufficient vitamins to provide equivalent intake in CR and AL rats; PMI Feeds) at 1800 that was expected to result in a food consumption equal to ~60% of AL food intake. The duration of CR was 20 days.

On the day they were killed, animals (~5.5 mo old) were weighed, and between 1300 and 1800 they were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt). The order of anesthetization was randomized so that the mean time of tissue sampling did not vary between the groups. Blood, sampled by cutting the tip of the tail, was used for determination of plasma insulin concentration. Animal body weight was determined after removing the body of skin and fat, and between 1300 and 1800 they were anesthetized by 10.2±0.3°C on June 25, 2017 http://ajpendo.physiology.org/ Downloaded from
overnight at 4°C in PBST containing 1% milk, 0.02% NaN₃, containing 5% nonfat dry milk. Membranes were rinsed three times by autoradiography. The spots corresponding to the 3
products were resolved in a chloroform-methanol-water-organic phase containing the reaction products were spotted onto an aluminum-backed silica thin-layer chromatography plate and counted in a scintillation counter. Determination of IRS-1, IRS-2, and PI3K p85 subunit abundance. Gastrocnemius muscles were prepared and analyzed essentially as described by Goodyear et al. (19). A portion of the supernatant was analyzed for protein content with the BCA assay. The remaining supernatant was combined with Lammli buffer and boiled for 3–5 min. Duplicate aliquots (20 μg protein) from each sample were subjected to SDS-PAGE with a 7.5% resolving gel. Proteins were electrophoretically transferred to 0.45-μm Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for 1 h at room temperature with phosphate-buffered saline (PBST; 80 mM Na₂HPO₄, 100 mM NaCl, and 0.1% Tween-20, pH 7.5) containing 5% nonfat dry milk. Membranes were rinsed three times with PBST at room temperature and then incubated overnight at 4°C in PBST containing 1% milk, 0.02% NaN₃, and the appropriate primary antibody (IRS-1: 1,000-fold dilution of rabbit polyclonal antibody against 14 amino acid carboxy terminus, Upstate Biotechnology; PI3K: 1,667-fold dilution of antibody against the entire p85 subunit and SH2 carboxy terminus, Upstate Biotechnology; PI3K: 1,667-fold dilution of rabbit polyclonal antibody against 14 amino acid carboxy terminus, Upstate Biotechnology). Membranes were rinsed three times with room temperature with PBST containing 20 μg of phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) and 100 mM MgCl₂. The reaction was initiated at room temperature by the addition of 5 μl of phosphorylation mixture containing 880 μM ATP containing 20 μCi [γ−³²P]ATP (Amersham, Arlington Heights, IL). After 20 min with continuous vortexing, the reaction was stopped by the sequential addition of 8 N HCl and chloroform-methanol (1:1). The reaction mixture was vortexed for 5–10 min and then centrifuged at 2,000 × g for 1 min at room temperature. Blots were exposed to Hyperfilm (Amersham), and the IRS-1, PI3K, and IRS-2 were quantified with a Bio-Rad (Hercules, CA) scanning densitometer. For each protein, the amount of sample loaded on the gel was linearly related (r = 0.94) to the signal determined by densitometry. Values are expressed relative to the mean value for the AL group (mean AL = 100%). Statistics. Unless otherwise stated, comparisons between AL and CR groups were made with a two-tailed t-test; differences were accepted as statistically significant at the P ≤ 0.05 level.

RESULTS

Food intake and body weight. The CR rats ate 56% as much as their AL cohorts (Table 1). The body weight of CR animals was 81% of AL animals.

Blood glucose and plasma insulin. CR was associated with a reduction in blood glucose of 12% (Table 1). Plasma insulin concentration was 69% lower in CR vs. AL.

Basal and insulin- and hypoxia-stimulated 3-MG transport rates. Basal 3-MG transport was not significantly altered by CR (Table 2). CR led to marked differences in glucose transport compared with AL values with 200 µU/ml (Table 2) or 20,000 µU/ml (Fig. 1A) insulin. In contrast, hypoxia-stimulated glucose transport rates were similar between muscles from CR and AL animals (Table 2).

Muscle glycogen concentration. AL and CR epitrochlearis muscle glycogen concentrations were not significantly different (21.1 ± 2.4 and 18.2 ± 1.8 μmol/g, respectively).

Cell surface GLUT-4 content and glucose transport rate. The basal 3-MG transport rate (Fig. 1A) and basal cell surface GLUT-4 content (Fig. 1B) were not significantly altered by diet. Consistent with the results of earlier studies, insulin-stimulated 3-MG transport was significantly increased for muscles from CR vs. AL rats (Fig. 1A). Because we had demonstrated this CR-induced increase in the insulin-stimulated 3-MG trans-

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Food Intake, g/day</th>
<th>Blood Glucose, mg/dl</th>
<th>Plasma Insulin, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>334.9 ± 2.8</td>
<td>16.9 ± 0.1</td>
<td>103.4 ± 2.8</td>
<td>9.79 ± 1.37</td>
</tr>
<tr>
<td>CR</td>
<td>272.8 ± 2.9*</td>
<td>9.4 ± 0.1*</td>
<td>88.5 ± 2.2*</td>
<td>3.03 ± 0.35*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 8 rats/group for insulin and 44–64 rats/group for body weight, food intake, and blood glucose. *P < 0.05 for ad libitum fed (AL) vs. calorie restricted (CR).

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal</th>
<th>Insulin, 200 µU/ml</th>
<th>Hypoxia, 15 min</th>
<th>Hypoxia, 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>204 ± 33</td>
<td>210 ± 48</td>
<td>251 ± 46</td>
<td>323 ± 27</td>
</tr>
<tr>
<td>CR</td>
<td>195 ± 31</td>
<td>399 ± 65*</td>
<td>236 ± 37</td>
<td>341 ± 46</td>
</tr>
</tbody>
</table>

Values are means ± SE for 8–12 muscles. Epitrochlearis 3-O-methylglucose transport rate is expressed as nmol·g⁻¹·15 min⁻¹. *P < 0.05 for AL vs. CR muscles incubated under identical conditions.
port before processing the contralateral muscles for cell surface GLUT-4 content, we used a one-tailed t-test to compare the AL and CR groups for the amount of cell surface GLUT-4 in insulin-stimulated muscles. CR muscles incubated in the presence of 20,000 µU/ml insulin had significantly more GLUT-4 at the cell surface compared with muscles from the AL group (Fig. 1B). Because we measured cell surface GLUT-4 content and 3-MG transport rate in paired muscles from the same rats, we were able to evaluate GLUT-4 glucose transporter activity for each muscle pair: the 3-MG transport rate (nmol·g⁻¹·15 min⁻¹) determined for each muscle was divided by the cell surface GLUT-4 (counts·min⁻¹·g⁻¹) determined for the contralateral muscle. GLUT-4 glucose transporter activity was not significantly different for insulin-stimulated muscles from CR vs. AL rats (Fig. 1C). There was also no significant difference between AL and CR groups for GLUT-4 activity determined in basal muscles or for the insulin-stimulation above basal (determined by subtracting the mean value of the group for 3-MG transport and cell surface GLUT-4 under basal conditions, respectively, from the corresponding values for individual insulin-stimulated muscles, and calculating the 3-MG-to-GLUT-4 ratio with these Δ values; results not shown).

IRS-1-associated PI3K activity. IRS-1-associated PI3K activity in muscles incubated without added insulin did not differ between AL and CR groups (Fig. 2). As expected, muscles incubated with insulin had a higher...
IRS-1-associated PI3K activity compared with the basal controls. The time course for this increase was quite similar between the diet groups. A nonsignificant trend was observed for somewhat higher values (~30%) for insulin-stimulated muscles from the CR vs. AL rats at 8, 20, and 40 min, but at no time point was the difference significant. If the values for these three time points are pooled (n = 17–18 per diet group), the trend still does not attain statistical significance.

IRS-1, IRS-2, and PI3K p85 subunit abundance. In CR compared with AL rats, IRS-1 abundance was 50% lower in gastrocnemius muscles (P < 0.05; Fig. 3A). Muscle levels of IRS-2 (Fig. 3B) and the p85 subunit of PI3K (Fig. 3C) did not differ between the diet groups.

DISCUSSION

Altered energy availability is a matter of fundamental biological importance, and reduced calorie intake has profound effects on glucose homeostasis and insulin action. The results of this experiment provide important new insights regarding the mechanisms by which CR amplifies insulin stimulation of glucose transport. We have previously demonstrated that CR does not alter total GLUT-4 levels in epitrochlearis or gastrocnemius muscles (10). This observation suggested that CR enhances the ability of insulin to increase the number of glucose transporters at the cell surface and/or the activity of those glucose transporters on the cell surface (i.e., glucose flux per transporter at the surface). Our results indicate that the CR-induced enhancement in insulin-stimulated glucose transport is the result of more GLUT-4 transporters at the cell surface rather than enhanced GLUT-4 activity: the abundance of cell surface GLUT-4 content was increased in CR vs. AL muscles, and GLUT-4 activity (glucose transport rate divided by cell surface GLUT-4) was similar for CR compared with AL muscles. Insulin increases the glucose transport rate of adipocytes and myocytes by activating the redistribution of GLUT-4 glucose transporters from intracellular vesicles to the cell surface. In adipocytes, the insulin-mediated redistribution appears to be primarily the result of enhanced exocytosis of GLUT-4-containing vesicles (35). Our data indicate that the steady-state proportion of GLUT-4 residing in the cell surface of insulin-stimulated muscles is greater in CR vs. AL animals.

GLUT-4 is the predominant glucose transporter expressed by skeletal muscle, but a small amount of GLUT-1 is also present, primarily in the perineural sheaths (20). Therefore, total glucose transport equals the sum of GLUT-1-mediated and GLUT-4-mediated glucose transport. Because insulin induces little or no detectable translocation of GLUT-1 in skeletal muscle (17, 31), GLUT-1 is more important for basal than
insulin-stimulated glucose transport rate. Basal glucose transport and cell surface GLUT-4 content were virtually identical between the diet groups, suggesting little or no diet-related change in GLUT-1 function. In insulin-stimulated muscle, the abundance of GLUT-4 is more than fivefold that of GLUT-1 (31), and intrinsic activity of GLUT-4 is about threefold that of GLUT-1 (22). In this context, our calculated values of GLUT-4 activity (which were determined with total glucose transport rate) are probably slight overestimates, but this would be minimized by the increase in cell surface GLUT-4 in insulin-stimulated muscles, and it seems unlikely that GLUT-1 is quantitatively important for the CR-induced increase in insulin-stimulated glucose transport rate.

In light of the reduced concentrations of circulating glucose and insulin with CR, it was reasonable to suspect that muscle glycogen concentration would also be reduced. A reciprocal relationship between glucose transport rate and muscle glycogen concentration has been noted under some conditions (e.g., after exercise), so a decline in glycogen stores might be important for the CR-induced increase in glucose transport. However, glycogen concentration did not differ between the diet groups, demonstrating that the CR effect on glucose transport was not secondary to muscle glycogen depletion. The preservation of muscle glycogen in CR rats, despite moderately lower glycemia and markedly reduced insulinemia, was presumably due, at least in part, to an enhanced capacity for insulin-stimulated glucose transport.

In skeletal muscle, hypoxia stimulates glucose transport via a pathway that is distinct from that activated by insulin, as evidenced by the observations that the maximal effects of insulin and hypoxia on glucose transport are additive (8), and low concentrations of wortmannin, which can entirely prevent insulin-stimulated glucose transport, do not affect hypoxia-stimulated glucose transport (30, 43). We evaluated hypoxia-stimulated glucose transport because it seemed possible that CR might induce a nonspecific alteration (e.g., membrane fluidity) that would lead to enhanced activation of glucose transport by various stimuli. Our finding that CR enhances insulin-mediated, but not basal or hypoxia-stimulated, glucose transport indicates that CR acts specifically on the insulin-stimulated pathway. Type 2 diabetes also has a specific effect on the insulin-stimulated pathway but in the converse direction: muscle strips from people with type 2 diabetes were profoundly insulin resistant yet retained a robust stimulation of glucose transport by in vitro hypoxia (4).

In this context, it is interesting that a moderate reduction in calorie intake and/or increased physical activity is commonly recommended for treating individuals with insulin resistance (1, 2), and the benefits of these interventions are due, at least in part, to an increase in skeletal muscle glucose transport (7, 11). Our current results, taken together with the results of earlier studies of exercise, reveal the distinctive characteristics of the two interventions. Muscle glycogen depletion during vigorous exercise appears to be crucial for the insulin-independent, postexercise increase in glucose transport (24). By contrast, 20 days of CR did not elicit alterations in glycogen concentration or basal glucose transport. The specific enhancement in the activation of glucose transport via the insulin pathway after CR also differs from the postexercise condition, which is characterized by a nonspecific increase in susceptibility to activation by various stimuli, including in vitro hypoxia (9). Exercise and CR appear to enhance glucose transport by separate mechanisms, indicating that the combination of the two interventions might result in an additive increase in glucose disposal.

The specificity of the effect of CR on the insulin pathway suggested that CR influences the insulin signal transduction system. Brief CR does not alter the number, binding affinity, or tyrosine kinase activity of insulin receptors from rat skeletal muscle (5, 12). Accordingly, we focused on postreceptor insulin signaling (IRS-1-associated PI3K activity) and, consistent with other studies (19, 42, 46), found that insulin led to a substantial increase above basal values. Our novel findings were that CR altered neither the magnitude nor the time course for insulin’s stimulation of IRS-1-associated PI3K activity. Possible explanations for the CR-induced increase in insulin-stimulated glucose transport include the observations that 1) CR acts on a pathway that does not involve IRS-1-associated PI3K (e.g., IRS-2-associated PI3K), 2) CR amplifies steps distal to IRS-1-associated PI3K, or 3) CR alters the subcellular localization of a portion of the IRS-1-associated PI3K. Regarding the third possibility, there is evidence supporting the concept that the subcellular localization of the PI3K activity is functionally important for insulin signal transduction and action (23, 27). We focused on a maximally effective insulin concentration in this study, so our results do not address the possible influence of CR on insulin signaling with submaximally effective concentrations.

We also measured the abundance of three proteins that are instrumental for insulin signaling (IRS-1, IRS-2, and p85 PI3K). As in the epitrochlearis, insulin-stimulated glucose uptake by the gastrocnemius is increased above AL levels after brief CR (14), and we previously demonstrated that CR does not alter the GLUT-4 protein expression in either the gastrocnemius or epitrochlearis (10). The fiber-type composition of the gastrocnemius (74% fast glycolytic, FG; 20% fast oxidative glycolytic, FOG; 6% slow oxidative, SO) is almost identical to the epitrochlearis (75% FG; 17% FOG; 8% SO), and each muscle corresponds quite closely to the average fiber-type composition (76% FG; 19% FOG; 5% SO) of all the muscles in the rat hindlimb (3, 37). The CR-induced increase in glucose uptake by the gastrocnemius (14) also corresponds with the effect of CR on a large portion of the total muscle mass, as evidenced by the induction of substantial increases in whole body glucose clearance (34) and glucose uptake by the perfused hindlimb (25). Taken together, these results indicate that the gastrocnemius and epitrochlearis are
representative muscles for evaluating the effects of CR. There was no effect of diet on muscle concentration of the p85 subunit of PI3K, but the abundance of IRS-1 protein was substantially lower in muscles from CR compared with AL rats. It seems unlikely that a change in IRS-1 per se leads to increased insulin action; e.g., mice heterozygous for the IRS-1 null allele have an ~50% reduction in muscle IRS-1 (comparable to the difference between AL and CR), but the heterozygous (IRS-1 +/−) mice are not more insulin sensitive than wild-type (IRS-1 +/+) littermates (6).

Recent research has identified a family of IRS proteins, including IRS-1, IRS-2, IRS-3, and IRS-4. Information about the expression of IRS-4 by skeletal muscle has apparently not been published, but IRS-3 is reportedly not expressed by skeletal muscle (36). IRS-1 and IRS-2 are members of the IRS family known to be expressed by skeletal muscle (33). IRS-2 is relevant to glucoregulation, as evidenced by the finding that IRS-2 null mice become diabetic and insulin resistant (41). Accordingly, we measured the abundance of IRS-2 in the gastrocnemius (which has a representative fiber-type composition and which exhibits increased insulin-stimulated glucose uptake with brief CR) and found that the quantity of IRS-2 was similar in muscles from AL and CR rats, arguing against the possibility that altered IRS-2 expression accounts for the increased insulin action. It remains possible that a qualitative change in IRS-2 (e.g., phosphorylation status, subcellular distribution, and/or association with PI3K) plays a role in the increased glucose transport.

In conclusion, the CR-induced enhancement of insulin action in skeletal muscle is well-known, but few studies have addressed the cellular and molecular processes underlying this fundamentally important aspect of physiology. Our data provide new insights into the mechanism for increased glucose transport in skeletal muscle of CR animals, including that this effect is independent of 1) a change in glycogen concentration; 2) an altered magnitude or time course of IRS-1-PI3K activity; 3) an increased expression of IRS-1, IRS-2, or p85 PI3K proteins; and 4) an increased GLUT-4 activity. Furthermore, our data demonstrated that the CR effect on glucose transport is specific to the insulin pathway and is attributable to an increase in the amount of GLUT-4 translocated to the cell surface in response to insulin stimulation.

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Received 20 May 1998; accepted in final form 13 August 1998.

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