Calorie restriction increases the ratio of phosphatidylinositol 3-kinase catalytic to regulatory subunits in rat skeletal muscle

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MCCURDY, Carrie E., Robert T. Davidson, and Gregory D. Cartee. Calorie restriction increases the ratio of phosphatidylinositol 3-kinase catalytic to regulatory subunits in rat skeletal muscle. Am J Physiol Endocrinol Metab 288: E996–E1001, 2005. First published December 21, 2004; doi:10.1152/ajpendo.00566.2004.—Calorie restriction (CR; 60% of ad libitum (AL) intake) improves insulin-stimulated glucose transport, concomitant with enhanced phosphorylation of Akt. The mechanism(s) for the CR-induced increase in Akt phosphorylation of insulin-stimulated muscle is unknown. The purpose of this study was to determine whether CR increased the ratio of catalytic to regulatory subunits favoring enhanced phosphatidylinositol (PI) 3-kinase signaling, which may contribute to increases in Akt phosphorylation and glucose transport in insulin-stimulated muscles. We measured the PI 3-kinase catalytic ($p110_\beta$, $p50_\alpha$, and $p55_\alpha$) and catalytic ($p110$) subunits abundance in skeletal muscle from male F344/N rats after 8 wk of AL or CR treatment. In CR compared with AL, regulatory isoforms, $p50_\alpha$ and $p55_\alpha$ abundance were $\sim40\%$ lower ($P<0.01$) with unchanged $p85_\alpha/\beta$ levels. There was no diet-related change in catalytic subunit abundance. Despite lower IRS-1 levels ($\sim35\%$) for CR vs. AL, IRS-1-p110 association in insulin-stimulated muscles was significantly ($P<0.05$) enhanced by $\sim50\%$. Downstream of PI 3-kinase, CR compared with AL significantly enhanced Akt serine phosphorylation by 1.5-fold higher ($P=0.01$) and 3-O-methylglucose transport by $\sim20\%$ in muscles incubated with insulin. The increased ratio of PI 3-kinase catalytic to regulatory subunits favors enhanced insulin signaling, which likely contributes to greater Akt phosphorylation and improved insulin sensitivity associated with CR in skeletal muscle.

Insulin sensitivity; p85 subunits; p55α; p50α; p110

MODERATE CALORIE RESTRICTION [CR; 25–40% reduction below ad libitum (AL) intake] is an effective treatment for increasing insulin sensitivity, in part, through enhanced insulin-stimulated glucose uptake in skeletal muscle (5, 8–10, 13). Recently, we found that CR compared with AL significantly increased the phosphorylation of Akt2 (24), a protein that is important in insulin-stimulated glucose uptake (14, 17, 19). Moreover, we found that enhanced Akt2 phosphorylation paralleled CR-mediated increases in insulin-stimulated glucose uptake (23, 24).

Phosphatidylinositol (PI) 3-kinase, an upstream regulator of Akt, is an obvious candidate for mediating the CR-induced increase in insulin-stimulated Akt phosphorylation in skeletal muscle. PI 3-kinase activation is required for insulin-stimulated glucose transport (6). With insulin stimulation, the class IA PI 3-kinases function as heterodimers consisting of a regulatory and catalytic subunit. The regulatory subunit acts as a bridge that couples tyrosine-phosphorylated IRS proteins with the catalytic subunit. Four isoforms of the regulatory subunit (p85α and its gene splice variants, p50α, p55α, and p85β) (1, 16, 25) and two isoforms of the catalytic subunit (p110α and p110β) have been identified in skeletal muscle (2, 3). In mouse embryonic fibroblasts, the regulatory subunits have been shown to be in stoichiometric excess (2:1) to the catalytic subunit resulting in a pool of regulatory subunit monomers not associated with a catalytic subunit (29, 30). These regulatory subunit monomers can also bind to phosphorylated IRS proteins. However, binding of the regulatory monomer to phosphorylated IRS blocks access to regulatory-catalytic heterodimers and thereby interferes with further insulin signaling. Optimal PI 3-kinase signaling through the PI 3-kinase pathway depends on a critical molecular balance between the regulatory and catalytic subunits. Therefore, the ratio of catalytic to regulatory subunits is important for determining PI 3-kinase function and insulin sensitivity. For example, insulin sensitivity is improved when the ratio of catalytic to regulatory subunit is increased. The increased catalytic to regulatory subunit ratio and subsequent enhancement in insulin sensitivity have been observed in isoform-specific (p85α, p85β, p50α, and p55α) knockout mice (7, 28, 31), where the abundance of the regulatory subunit pool was lowered and in 3T3-L1 adipocytes that overexpress the p110β catalytic subunit (2).

With CR in rats, we repeatedly observed a trend for a statistically nonsignificant increase in insulin-stimulated, IRS-1-associated PI 3-kinase activity in skeletal muscle (8, 9, 11). Because we consistently saw this trend in IRS-1 PI 3-kinase activity by an in vitro kinase assay, we now investigated the effect of CR on PI 3-kinase by a different method of analysis. The primary aim of the present study was to determine if CR increased the ratio of catalytic to regulatory subunits favoring enhanced PI 3-kinase signaling in insulin-stimulated muscles. To investigate this question, we measured PI 3-kinase regulatory and catalytic subunit abundance in isolated skeletal muscles after 8 wk of either CR or AL feeding. We also measured levels of IRS-1-associated p110, Akt serine phosphorylation, and insulin-stimulated 3-O-methylglucose transport in epitrachlearis muscles. We hypothesized that CR compared with AL would increase the ratio of PI 3-kinase catalytic to regulatory subunits, which would in turn favor increased IRS-1-

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p110 association in insulin-stimulated muscles. Such an increase in catalytic to regulatory subunits may be responsible, in part, for increased Akt phosphorylation and ultimately enhanced insulin sensitivity associated with CR.

**Materials and Methods**

**Antibodies.** Rabbit polyclonal anti-P1 3-kinase p85 (p85\(^{\text{AP}}\)) antibody (catalogue no. 06 –195) and rabbit polyclonal anti-IRS-1 antibody (no. 06 –248) were purchased from Upstate Biotechnology (UBI; Charlottesville, VA). Agarose-conjugated antibodies for p85\(^{\text{AP}}\) (UBI no. 16 –107) and IRS-1 (UBI no. 16 –146) were used for immunoprecipitation assays. Abundance of the catalytic subunit of PI 3-kinase was measured using rabbit polyclonal p110 antibody that recognizes all p110 isoforms (Santa Cruz Biotechnology, Santa Cruz, CA; no. sc-14502). A rabbit polyclonal phospho-Akt(Ser473) antibody that recognizes all serine-phosphorylated Akt isoforms was used to evaluate activation of Akt (Cell Signaling Technology, Beverly, MA; no. 9271).

**Animal care and treatment.** Animal care and use were approved by the University of Wisconsin-Madison Animal Care and Use Committee. Three-month-old male Fisher 344 × Brown Norway rats were obtained through the National Institute on Aging from Harlan Sprague Dawley (Indianapolis, IN). After arriving at the University of Wisconsin-Madison animal facility, rats were individually housed in wire-bottomed cages and maintained on a 12:12-h light-dark cycle with free access to rat chow (rodent diet no. 8604, Harlan Teklad, Madison, WI) and water during a 7-day acclimation period. After the acclimation period, baseline food intake was determined for each rat by measuring daily AL food intake over a 7-day period, correcting for spillage. Rats were then randomly assigned to an AL control group or a calorie-restricted (CR) treatment group. Rats in the CR group received 60% of their individual baseline food intake for the duration of the study. The CR dietary treatment lasted for 8 wk. For the first 4-wk period, CR rats were fed 60% of baseline intake at 1900 on a once-daily feeding schedule, whereas the AL group had free access to food. For the subsequent 4-wk period, all rats were fed on a time-matched feeding protocol as previously described (8). Briefly, all rats were given a portion of their food allotment in three 1.5-h periods (35% at 0700, 25% at 1500, and 40% at 2000). At each 1.5-h time period, the portion of food given was completely consumed. During the time-matched protocol, the AL group received 100% of their baseline food intake, whereas the CR group continued receiving 60% of their individual baseline intake. The purpose of matching the time of feeding for AL and CR was to evaluate the effects of reduced food intake, independent of temporal differences in food consumption. Because the time-matched feeding protocol is very time and labor intensive, we used this feeding schedule only during the final 4-wk period, a sufficient amount of time to reach a steady state. Food intake, independent of temporal differences in food consumption.

**Muscle preparation.** A portion of frozen muscles, to be used for immunoblotting, was homogenized on ice in glass-on-glass homogenizing tubes (Kontes, Vineland, NJ) with 0.5 ml of ice-cold homogenizing buffer as previously described (24). Muscle homogenate was transferred to flat-bottom microfuge tubes and solubilized for 1–2 h at 4°C with constant end-over-end rotation. Homogenate was centrifuged at 12,000 g for 12 min at 4°C. Aliquots of supernatant were frozen at −80°C for immunoblotting.

**Immunoprecipitation.** For immunoprecipitation (IP), samples were initially incubated with antibody overnight at 4°C with constant end-over-end rotation. Specifically, the PI 3-kinase regulatory subunits (p85\(^{\text{AP}}, p50^{\text{AP}}, p55^{\text{AP}}, \text{and } p85^{\text{AP}}\) were isolated from plantaris supernatant by IP with p85\(^{\text{AP}}\) agarose-conjugated antibody, which recognizes the common NH\(_2\)-terminal SH2 domain of all of the regulatory subunits. The agarose-conjugated anti-p85\(^{\text{AP}}\) (20 μl of 50% slurry) was incubated with 500 μg of total protein from plantaris supernatant. For p110 IP, 2 μg of rabbit anti-p110 antibody was incubated with 250 μg of total protein from epiterophicleirs supernatant. After initial antibody incubation, 40 μl of 50% slurry of protein A-agarose beads were added to the lysate/antibody mix and rotated at 4°C for 2 h. Protein A-agarose beads were isolated by centrifugation and washed three times in PBS with 1% Igepal and 0.1 mM Na\(_2\)VO\(_4\). Antigens were eluted from the beads with 60 μl 2× SDS loading buffer and were boiled for 5 min before SDS-PAGE as described below.

**Immunoblot assay.** IRS-1 abundance was measured in plantaris supernatant (30 μg of total protein), and Akt serine phosphorylation was assessed in supernatant from epiterophicleirs muscles incubated with and without insulin (60 μg of total protein). Samples were separated by 7.5% SDS-PAGE and transferred to nitrocellulose at 200 mA at 4°C. Membranes were blocked with 5% blocking grade milk (Bio-Rad, Hercules, CA) in PBS with 0.1% Tween 20. Membranes were initially incubated with antibody overnight at 4°C in PBS with 0.1% Tween 20 or Tris-buffered saline plus 0.1% Tween 20 for 1 h at room temperature and then incubated with primary antibody overnight at 4°C (1:5,000 for anti-p85\(^{\text{AP}}\) and anti-IRS-1; 1:1,000 for anti-IRS-1 and anti-phospho-Akt). Membranes were then incubated in horseradish peroxidase-conjugated secondary antibody (UBI) and developed with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Bands were quantitated by densitometry using Molecular Analyst software (Bio-Rad). The apparent molecular masses of immunoreactive proteins were determined based on comparisons with molecular mass standards. Band densities on each blot were expressed relative to the mean of the AL group (insulin-stimulated AL values for phosphorylation data) that had been adjusted to equal 1.0.

**Statistical analysis.** Two-way ANOVA with insulin and diet as the main effects was used to determine significant differences for 3MG transport, IRS-1-p110 association, and Akt serine phosphorylation. Student’s t-test was used to determine significant differences between AL and CR for immunoblot abundance data (p85α/β, p55α, p50α, 32) and respond similarly to CR (23, 33). Accordingly, for immunoblot analyses that did not require in vitro incubations of muscles to assess insulin activation (PI 3-kinase subunit and IRS-1 abundance), plantaris muscles were also dissected out, immediately freeze-clamped using tongs cooled in liquid nitrogen, and stored at −80°C for later analysis.

**3-O-methylglucose transport assay.** Dissected epiterophicleirs muscles were incubated (30°C), with gentle agitation, in flasks of continuously oxygenated (95% O\(_2\)-5% CO\(_2\)) Krebs-Henseleit incubation buffer (KHB; 0.1% BSA, 2 mM sodium pyruvate, 6 mM mannitol) with one muscle from each pair incubated in KHB without insulin (basal) and the contralateral muscle incubated in KHB with insulin (3 nM). After 30 min of incubation, muscles were transferred to flasks containing KHB-BSA with 8 mM 3-O-[\(^{3}H\)]methylglucose (3MG; 0.25 mCi/μmol; New England Nuclear Life Science products, Boston, MA), 2 mM [\(^{14}C\)]mannitol (0.1 mCi/μmol; Amershams Pharmaceuticals), and with the same insulin concentration as in the first incubation. After 15 min, muscles were blotted, trimmed, freeze-clamped using tongs cooled in liquid nitrogen, and stored at −80°C for later analysis. A portion of the muscle was homogenized in 0.3 N perchloric acid and 3MG transport rate was determined as previously described (4).

**Statistical analysis.** Two-way ANOVA with insulin and diet was used to determine significant differences for 3MG transport, IRS-1-p110 association, and Akt serine phosphorylation. Student’s t-test was used to determine significant differences between AL and CR for immunoblot abundance data (p85α/β, p55α, p50α, 32) and respond similarly to CR (23, 33). Accordingly, for immunoblot analyses that did not require in vitro incubations of muscles to assess insulin activation (PI 3-kinase subunit and IRS-1 abundance), plantaris muscles were also dissected out, immediately freeze-clamped using tongs cooled in liquid nitrogen, and stored at −80°C for later analysis.
RESULTS

3-O-methylglucose transport. The rate of 3MG transport was determined in paired epitrochlearis muscle incubated without or with insulin (3 nM; Fig. 1). As expected, insulin significantly increased 3MG transport above basal in AL and CR groups, and CR vs. AL further enhanced 3MG transport by ~20% in muscle incubated with insulin ($P < 0.05$). Basal 3MG transport was not different for AL and CR.

IRS-1 abundance. IRS-1 abundance was significantly ($P < 0.05$) lower by 35% with CR compared with AL in the plantaris muscle (Fig. 2).

PI 3-kinase regulatory subunit abundance. The abundance of the PI 3-kinase regulatory subunits (p85α, its splice variants p55α, and p50α, and p85β) was measured for AL and CR in plantaris muscle (Fig. 3). There was no effect of diet on p85α/p85β abundance. However, CR compared with AL significantly reduced both p55α ($P = 0.02$) and p50α ($P = 0.003$) abundance by ~40%. The total regulatory pool (sum of p85α/p85β, p55α, and p50α) was significantly reduced by ~25% for CR vs. AL ($P = 0.0005$).

IRS-1-associated p110 abundance. The total abundance of the p110 catalytic subunit was not significantly different in epitrochlearis muscle from AL and CR (Fig. 4). In epitrochlearis stimulated with insulin compared with basal, there was a significant increase ($P < 0.005$) in the amount of IRS-1.
associated with p110 regardless of diet (Fig. 5). For CR vs. AL, the amount of IRS-1 associated with p110 was further enhanced 1.5-fold in muscle incubated with insulin (P = 0.02).

Akt serine phosphorylation. There was no effect of diet on basal Akt serine phosphorylation. Total Akt serine phosphorylation was significantly increased (P < 0.001) in epitrochlearis muscles incubated with insulin regardless of diet (Fig. 6). In insulin-stimulated muscles, CR compared with AL enhanced Akt serine phosphorylation ~1.5-fold (P = 0.003).

DISCUSSION

One of the hallmarks of CR is improved insulin-stimulated glucose transport in skeletal muscle. Changes in the ratio of the PI 3-kinase catalytic to regulatory subunit abundance can alter insulin signaling and impact insulin sensitivity (2, 22, 29, 30). In the present study, we found that CR decreased the expression of the PI 3-kinase regulatory p55α and p50α subunit isoforms, without altering the abundance of p85α/β regulatory or p110 catalytic subunits, thereby increasing the ratio of PI 3-kinase catalytic to regulatory subunit abundance in skeletal muscle. This shift in the molecular balance (i.e., the ratio of catalytic to regulatory subunits) of PI 3-kinase with CR was accompanied by an increase in amount of p110 associated with IRS-1 in muscles stimulated with insulin. Additionally, such CR-induced increases in insulin signaling were accompanied by increased insulin-stimulated serine phosphorylation of Akt and an enhancement in insulin-stimulated glucose transport.

Recently, we showed that Akt2 plays a critical role in mediating the effects of brief CR on insulin-stimulated glucose transport, as mice lacking Akt2 have little or no enhancement in insulin-stimulated 2-DG in skeletal muscle in response to CR (23). We repeatedly observed a two- to threefold increase in Akt2 serine phosphorylation in insulin-stimulated muscle from CR compared with AL animals, and these increases have consistently corresponded with the CR-induced improvement in insulin-stimulated glucose transport (23, 24). The mechanism for the CR-induced increase in Akt phosphorylation is not known. However, we consistently saw a statistically nonsignificant trend for an increase in IRS-1-associated PI 3-kinase activity with CR compared with AL, suggesting a possible role for PI 3-kinase in the CR-mediated increase in Akt. The purpose of the present study was to evaluate whether CR alters PI 3-kinase subunit abundance as a potential mechanism to enhance downstream signaling at Akt.

An increase in the ratio of the catalytic to regulatory PI 3-kinase subunit abundance can lead to improvements in insulin sensitivity (2, 22, 29, 30). The PI 3-kinase regulatory subunits have a dual role in modulating insulin signaling. As part of a heterodimer with the catalytic subunit, the regulatory subunits couple the catalytic subunits with IRS proteins, but in the monomeric state, they block access of the functional heterodimers to tyrosine-phosphorylated IRS proteins and thereby block PI 3-kinase signaling. In transgenic mice lacking p85α (28), p85β (31), or p55α/p50α (7), the ratio of PI 3-kinase catalytic to regulatory subunits is increased, leading to enhanced whole body insulin sensitivity. Additionally, troglitazone treatment in diabetic patients significantly increased p110β abundance without changing regulatory subunit abundance in skeletal muscle, concomitant with improved insulin sensitivity (18). These studies support the hypothesis that an increase in the ratio of catalytic to regulatory subunit abundance with CR may contribute to the enhancement in insulin sensitivity.

In this study, the decreased p55α and p50α with CR compared with AL led to a significant ~25% decrease in the total regulatory pool in skeletal muscle. This was accompanied by an ~30% increase in glucose transport in insulin-stimulated skeletal muscle from CR rats. Similar results were observed in a study by Ueki et al. (31) in which loss of p85β resulted in an ~30% decrease in the total regulatory subunit pool in skeletal muscle and led to ~25% increase in whole body insulin sensitivity. The shift in the ratio of PI 3-kinase catalytic to regulatory subunit abundance leads to a proportional change in
downstream insulin signaling and insulin sensitivity (12, 21, 22, 26, 28). Therefore, the decrease in the total regulatory pool size with CR would be predicted to contribute to the increase in Akt phosphorylation and glucose uptake in muscles incubated with insulin.

Along with the increase in the ratio of catalytic to regulatory subunits, IRS-1-p110 association was significantly increased (~1.5-fold with CR compared with AL, which would be suggestive of enhanced IRS-1 PI 3-kinase function. We consistently observed a trend for a statistically nonsignificant (~45%) increase in insulin-stimulated, IRS-1-associated PI 3-kinase activity in rat skeletal muscle with 20-day CR vs. AL. To facilitate statistical analysis of pooled data from the two previous CR experiments with isolated rat epitrochlearis muscle (8, 9), we expressed IRS-1-associated PI 3-kinase activity as a ratio of values in insulin-treated muscles divided by the average value in muscles incubated without insulin. When the pooled data were compared using a t-test, IRS-1-associated PI 3-kinase activity was significantly (P = 0.02) greater for CR (5.5 ± 0.7, n = 24) compared with AL (3.5 ± 0.4, n = 26). The period of CR was longer in the current experiment (8 wk) compared with these earlier studies (20 days), but the relative magnitudes of CR-induced increases in glucose transport (~20 to 30%) (8) and total Akt serine phosphorylation (35–50%) (24), as well as the decrease in IRS-1 abundance (~35–50%) (9), have been quite similar between the two durations of CR. Given that increased IRS-1-associated p110 would be expected to lead to increased PI 3-kinase activity, the simplest interpretation of the combined results is that the CR-induced elevation in IRS-1-p110 in the current study is a reasonable explanation for the apparent CR-induced increase in IRS-1-PI 3-kinase activity revealed by analyzing pooled data from previous experiments.

In this study, we measured IRS-1-p110 association and regulatory subunit abundance in different muscles (epitrochlearis and plantaris, respectively). The fiber-type compositions for the plantaris (15, 31, and 54% for type I, type IIa, and type IIb, respectively) and epitrochlearis (8, 17, and 75% for type I, type IIa, and type IIb, respectively) are roughly similar (15, 32). We found that the epitrochlearis and plantaris muscles respond similarly to CR. The ~35% CR-induced decline in IRS-1 abundance for the plantaris compares with the ~38% decline in the epitrochlearis for CR vs. AL (Dean DJ and Cartee GD, unpublished observations). The lack of a change in p85 abundance in the plantaris with CR is consistent with our previous results in the epitrochlearis (Dean DJ and Cartee GD, unpublished observations). CR also does not alter the abundance of SH2 domain-containing inositol phosphatase of either the plantaris or epitrochlearis (23). In vivo glucose uptake was increased in both the plantaris and epitrochlearis for CR vs. AL rats (33). In this context, it seems reasonable to suspect that the CR-induced increase in IRS-1 association with p110 in insulin-stimulated epitrochlearis is due to decreased expression of p55α and p50α isoforms with CR compared with AL.

In conclusion, this study demonstrated that CR results in a decrease in the total PI 3-kinase regulatory subunit pool in rat skeletal muscle. The levels of PI 3-kinase p55α and p50α isoforms were significantly lower for CR compared with AL with no change in the abundance of the p85α/β regulatory subunit or p110 catalytic subunit. The resultant increase in the ratio of catalytic to regulatory subunits with CR is a likely explanation for the CR-induced increase in IRS-1-p110 interaction in insulin-stimulated muscle, which in turn would be predicted to lead to the increase in Akt serine phosphorylation found for CR compared with AL muscle stimulated by insulin. The results of this study reveal a novel aspect of the mechanism for the CR-induced increase in skeletal muscle insulin signaling and the concomitant increase in insulin-stimulated glucose transport. These molecular adaptations in skeletal muscle are likely to be important for the improved whole body insulin sensitivity that is a hallmark of CR.

GRANTS

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REFERENCES


