Calorie Restriction Increases Insulin-Stimulated Glucose Transport in Skeletal Muscle From IRS-1 Knockout Mice

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Calorie restriction (CR), even for brief periods (4–20 days), results in increased whole-body insulin sensitivity, in large part due to enhanced insulin-stimulated glucose transport by skeletal muscle. Evidence suggests that the cellular alterations leading to this effect are postreceptor steps in insulin signaling. To determine whether insulin receptor substrate (IRS)-1 is essential for the insulin-sensitizing effect of CR, we measured in vitro 2-deoxyglucose (2DG) uptake in the presence and absence of insulin by skeletal muscle isolated from wild-type (WT) mice and transgenic mice lacking IRS-1 (knockout [KO]) after either ad libitum (AL) feeding or 20 days of CR (60% of ad libitum intake). Three muscles (soleus, extensor digitorum longus [EDL], and epitrochlearis) from male and female mice (4.5–6 months old) were studied. In each muscle, insulin-stimulated 2DG uptake was not different between genotypes. For EDL and epitrochlearis, insulin-stimulated 2DG uptake was greater in CR compared to AL groups, regardless of sex. Soleus insulin-stimulated 2DG uptake was greater in CR compared with AL in males but not females. The diet effect on 2DG uptake was not different for WT and KO animals. Genotype also did not alter the CR-induced decrease in plasma constituents (glucose, insulin, and leptin) or body composition (body weight, fat pad/body weight ratio). Consistent with previous studies in rats, IRS-1 protein expression in muscle was reduced in WT-CR compared with WT-AL mice, and muscle IRS-2 abundance was unchanged by diet. Skeletal muscle IRS-2 protein expression was significantly lower in WT compared with KO mice. These data demonstrate that IRS-1 is not essential for the CR-induced increase in insulin-stimulated glucose transport in skeletal muscle, and the absence of IRS-1 does not modify any of the characteristic adaptations of CR that were evaluated. *Diabetes* 48:1930–1936, 1999

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AL, ad libitum; ANOVA, analysis of variance; CR, calorie restriction; 2DG, 2-deoxyglucose; EDL, extensor digitorum longus; HZ, heterozygous; IRS, insulin receptor substrate; IRTK, insulin receptor tyrosine kinase; KHB, Krebs-Henseleit buffer; KO, knockout; PI3K, phosphatidylinositol-3-kinase; WT, wild-type.

Impaired insulin-stimulated glucose clearance is a good predictor of future precipitation of type 2 diabetes in humans (1). Peripheral insulin resistance is thus considered a primary defect in the pathogenesis of glucose intolerance, and as such represents a critical point of intervention for preventing the development of glucose intolerance and overt diabetes.

Many investigations into the development of insulin resistance have utilized models of overnutrition, such as obese humans and rodent models of obesity and type 2 diabetes induced by hyperphagia or high-fat feeding. Chronic negative energy balance results in the converse physiologic state, one of increased peripheral insulin sensitivity and improved whole-body glucose homeostasis. Indeed, mild to moderate weight loss with moderate reduction in calorie intake are among the nutrition recommendations for patients with type 2 diabetes (2). Calorie restriction (CR), even when the obese condition persists, improves whole-body glucose homeostasis (3). The CR-induced improvement in metabolic control is due in large part to increased muscle glucose utilization (3).

Skeletal muscle insulin resistance is present in virtually all patients with type 2 diabetes (4) and is common in obesity. Muscle is quantitatively the most important peripheral tissue for insulin-stimulated glucose clearance (5). Furthermore, glucose transport appears to be the rate-limiting step for muscle glucose utilization (6) and evidence suggests that the decrease in muscle glucose utilization in insulin resistance is secondary to a primary defect in transport (7). Thus, we have focused on elucidating the cellular and molecular adaptations that result in the insulin-sensitizing effect of CR on this fundamentally important process, glucose transport.

The signaling events involved in increasing glucose transport by muscle in response to insulin stimulation are incompletely defined. However, several cellular events are known to occur (8). Insulin binding activates the insulin receptor tyrosine kinase (IRTK), resulting in autophosphorylation of specific tyrosine residues on the intracellular subunit of the receptor, which further increases IRTK toward exogenous substrates, such as the insulin receptor substrate (IRS) family of proteins. Of the four putative IRSs, IRS-1 and -2 are expressed in skeletal muscle. IRSs serve as docking proteins to which diverse signaling proteins bind in order to affect the many biological responses to insulin. Binding of phosphatidylinositol-3-kinase (PI3K) to IRS-1/2 activates the lipid kinase, resulting in the phosphorylation of the 3′ hydroxyl group on the inositol ring of various inositol

phosphates. PI3K activity is essential for insulin-stimulated glucose transport (9).

The enhanced insulin action in muscle in response to CR occurs rapidly, before large changes in body composition. In obese humans with type 2 diabetes, the improvement in insulin sensitivity after only 7 days of CR was similar to the further improvements acquired after 3 additional months of reduced calorie intake that resulted in considerable weight loss (10). Similarly, a substantial portion of the increase in muscle insulin–mediated glucose transport that occurs in rats after long-term (months) CR is acquired within days (4–20) of initiating a 25–40% reduction in calorie intake (11,12).

Brief CR does not alter the number, binding affinity, or tyrosine kinase activity of insulin receptors in rat skeletal muscle (13). However, the effect of CR on glucose transport appears to be specific to the insulin-mediated pathway, as evidenced by the observation that CR does not enhance the activation of glucose transport by an insulin-independent stimulus (in vitro hypoxia) (14). CR does not alter total GLUT4 protein expression in skeletal muscle (15); rather CR increases the amount of GLUT4 in the cell surface membranes in insulin-stimulated muscle (14). Given the above findings, and the suggestion that the cellular defect(s) resulting in insulin resistance is distal to insulin binding to its receptor, it was logical to suspect that CR influences postreceptor steps in insulin signaling.

The most proximal steps in the signaling cascade to the IRTK are the IRSs. We recently found that brief CR does not increase the amount of IRS-1–associated PI3K activity in skeletal muscle (14). This result does not eliminate the possibility that CR alters some other aspect of IRS-1 function (e.g., altering subcellular location of IRS-1), but it did raise the possibility that the influence of CR was mediated by IRS-1–independent mechanisms. Therefore, the primary aim of this study was to determine if IRS-1 is essential for the insulin-sensitizing effects of CR. Toward that end, we evaluated the effect of brief CR (20 days of consuming 60% of ad libitum intake) on insulin-stimulated glucose transport in isolated skeletal muscle from wild-type mice and transgenic mice lacking IRS-1. To gain insight into the role of IRS-1 in other metabolic consequences of moderate CR, we also assessed glycemia, insulinemia, leptinemia, and adipose tissue mass.

RESEARCH DESIGN AND METHODS

Animal breeding and care. Male mice heterozygous (HZ) for the null and intact IRS-1 alleles (16) were bred with female C57Bl/6J (Jackson Labs, Bar Harbor, ME) mice to produce an F1 generation consisting of wild-type (WT) mice homozygous for the intact IRS-1 allele, and HZ mice. At 3 weeks of age, tail tips (~1 cm) were biopsied from weanlings for DNA extraction (DNAzol; Molecular Research Center, Cincinnati, OH) and genotype was determined by polymerase chain reaction analysis as previously described (16).

A generation of F2 mice was produced by interbreeding HZ F1 males and females. F2 offspring were genotyped as above to identify WT and HZ mice, and knockouts (KO), mice null for the IRS-1 allele. Animals were given ad libitum access to food (PMI 5001; PMI Feeds, Richmond, IN) and water.

20 days calorie restriction. Males and females were used in the following study. WT controls and KO shared at least one parent. Six weeks before the experiment, mice were singly housed in wire-bottom cages (29–30°C, 25–35% humidity, 12:12 h light-dark cycle, with lights off at 1800). WT and KO mice were randomly assigned to two groups: ad libitum–fed (AL) and 20-day CR. Baseline daily food consumption was measured by weighing the food provided and correcting for food not eaten, including spillage. For the 20 days of CR, each CR mouse was provided with an allotment of food equal to 60% ad libitum (baseline) consumption. CR mice were provided with food between 1730–1800. Mice were 4.5–6 months old at the end of the study.

On the day of the isolated muscle experiment, AL animals were allowed access to food and all animals had free access to water. Experiments began at 1400. Mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). Blood was drawn with EDTA-treated capillary tubes via retro-orbital sinus. Soleus, extensor digitorum longus (EDL), and epitrochlearis muscles were rapidly dissected out for in vitro incubation. Gastrocnemius muscles were then rapidly dissected and freeze-clamped. All tissues were stored at –80°C until analysis. Retroperitoneal fat pads were carefully dissected and weighed.

2-Deoxyglucose transport measurement. Immediately upon dissection from the animal, muscles were placed in flasks of oxygenated Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 2 mmol/l Na-pyruvate, and 6 mmol/l mannitol and insulin at one of the following concentrations: none, a high physiologic level (0.6 nmol/l), or a maximally effective level (12 nmol/l). One muscle from each animal was used for determination of basal glucose uptake (no insulin), while the contralateral soleus and EDL were incubated with 12 nmol/l insulin and epitrochlearis were incubated with 0.6 nmol/l insulin (Humulin R; Lilly, Indianapolis, IN). Flasks were gently agitated in a shaking water bath (37°C) and continuously gassed with 95% O_2/5% CO_2. After 30 min, muscles were transferred to flasks of KHB-BSA containing 1 mmol/l [^{3}H]-2-deoxyglucose (2DG) (2 mCi/mmol) and 9 mmol/l [^{14}C]-mannitol (0.022 mCi/mmol) (ARC, St. Louis, MO) and insulin levels identical to those in the first incubation. After 20 min, muscles were blotted on filter paper, trimmed, and freeze-clamped. Muscles were stored at –80°C until further analysis.

Determination of 2DG transport rate. Frozen muscles were rapidly weighed and homogenized in ice cold 0.3N perchloric acid. Uptake of 2DG was calculated as previously described (17), and is expressed as micromoles of 2DG per gram of wet weight muscle per 20 min.

Plasma analysis. Collected blood was spun at 10,000*g* for 10 min. Supernatant (plasma) was stored frozen at –20°C until analysis. Insulin was measured by RIA (Linco, St. Charles, MO). Glucose levels were determined by enzymatic (Trinder) assay (Sigma, St. Louis, MO). Leptin was assayed by ELISA (R&D Systems, Minneapolis, MN).

Muscle IRS-1 and IRS-2 abundance. Frozen gastrocnemius was homogenized with glass-on-glass tubes (Kontes, Vineland, NJ) at a dilution of 1:9 (wt:vol) in ice-cold buffer (50 mmol/l HEPES, 1% Triton-X 100, 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l sodium vanadate, and protease inhibitors: 10 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, 0.5 µg/ml pepstatin, 10 mmol/l EDTA). Homogenates were transferred to microfuge tubes and rocked end-over-end for 1 h at 4°C. Samples were spun at 35,000*g* for 1 h at 4°C. Supernatants were decanted into fresh tubes and an aliquot was analyzed by bicinchoninic acid (BCA) assay for total protein content (Sigma, St. Louis, MO).

For each sample, the volume of supernatant corresponding to 4 mg cellular protein was normalized to a total volume of 1 ml using homogenization buffer. Immunoprecipitation with 4 µg anti–IRS-1 antibody (UBI, Lake Placid, NY) was carried out according to manufacturer's instructions. Supernatants from IRS-1 immunoprecipitation were sequentially immunoprecipitated with anti–IRS-2 antibodies (UBI). Absence of cross-reactivity between anti–IRS-1 and anti–IRS-2 antibodies was confirmed by Western blotting, and efficiency of each precipitation was 100%, as confirmed by Western blot analysis of a second round of immunoprecipitation, which yielded no detectable IRS protein.

Immunoprecipitates were fractionated by 7.5% SDS-PAGE and blotted electrophoretically onto PVDF filter (Millipore, Bedford, MA). IRS protein was detected using the same primary antibody as in the immunoprecipitation and a horseradish peroxidase conjugated anti-rabbit-IgG secondary antibody (Amersham, Arlington Heights, IL). Protein bands were visualized by enzyme chemiluminescence (Amersham) and quantitated by densitometry (BioRad, Hercules, CA). The optical density of the IRS-1 or IRS-2 band of each sample is expressed as a proportion of the weighted mean density of bands corresponding to IRS-1 or IRS-2 for all samples within each gel.

Statistical analysis. Regardless of sex, the effects of diet and genotype were similar for most parameters (2DG uptake in soleus was the exception). Therefore, unless otherwise noted, data for males and females were pooled for statistical analysis. When the effects of two factors were analyzed, two-way analysis of variance (ANOVA) was used, with dietary treatment (CR versus AL) and genotype (WT versus KO) as main effects. For soleus 2DG uptake, data from each sex were analyzed separately by two-way ANOVA. Because plasma leptin in several samples from the CR mice was below the detection limit of the assay, statistical analysis was by nonparametric two-way ANOVA on all data, assigning all samples below the limit of detection of the assay the same rank. When data were compared between two groups (e.g., IRS-1 was present only in WT mice), *t* test was used for analysis (Sigma Stat; SPSS, Chicago). Statistical significance in all tests was set at $P \leq 0.05$.

RESULTS

Food intake. Baseline ad libitum daily food consumption did not differ between mice randomized to CR and AL dietary treatments. Over the 20-day feeding period, CR food intake

TABLE 1
Body weight in grams before and after 20 days of CR or AL feeding in mice homozygous for the intact (WT) or null (KO) IRS-1 alleles

	Day 0				Day 20			
	WT	KO	Effect	*P*	WT	KO	Effect	*P*
AL	28.7 ± 1.4	16.9 ± 0.8	D	NS	28.2 ± 1.3	16.8 ± 0.7	D	<0.001
CR	28.2 ± 1.3	16.8 ± 0.8	G	<0.001	23.5 ± 1.1	13.7 ± 0.6	G	<0.001
			D × G	NS			D × G	NS

Data are means ± SE. n = 11–13. Data were analyzed by two-way ANOVA: D, main effect of dietary treatment; G, main effect of genotype; D × G, interaction between main effects.

averaged 62.5 ± 1.4% of baseline ad libitum intake, and AL food intake did not change from baseline.

Body and tissue weights. KO mice were significantly smaller than WT mice ($P < 0.001$) (Table 1). Over the 20-day restriction period, CR mice lost on average 19.3 ± 0.71% of initial body weight. There was no difference in the relative amount of weight lost between KO and WT mice (18.5 ± 0.81 and 19.9 ± 1.1%, respectively). AL mice body weights did not change over the 20-day feeding period.

Retroperitoneal fat pad mass expressed as a percent of body weight was significantly decreased with 20-day CR ($P < 0.001$), and to a similar extent in both genotypes: AL-WT, 0.27 ± 0.04%; AL-KO, 0.22 ± 0.02%; CR-WT, 0.09 ± 0.02%; CR-KO, 0.04 ± 0.02%. There was no significant effect of genotype on relative fat pad mass.

Plasma analysis. Plasma insulin levels (Table 2) were significantly decreased after 20-day CR ($P < 0.001$). KO mice had significantly higher insulin levels than WT mice ($P < 0.005$). Plasma glucose levels were significantly reduced in CR versus AL mice ($P < 0.001$) (Table 2). There was no difference in plasma glucose levels between KO and WT mice in either dietary treatment group. Plasma leptin was measured in all AL samples. For one CR-KO mouse there was insufficient plasma to perform the assay. In the remaining CR mice, leptin was present below detectable levels (0.44 ng/ml) in 5 of 13 WT, and 4 of 10 KO. Nonparametric analysis of ranks of all samples revealed a statistically significant main effect of diet ($P < 0.001$, CR less than AL) and genotype ($P < 0.05$, KO less than WT). There was no interaction between the effects of diet and genotype on plasma measures.

2DG uptake. Glucose uptake data from males and females were similar in the EDL and epitrochlearis; therefore, data were pooled. Basal 2DG uptake in EDL was not different between KO and WT mice, nor between AL and CR mice (Fig. 1). There was no main effect of genotype on 2DG uptake by EDL in the presence of insulin. CR significantly increased 2DG uptake in the presence of insulin ($P < 0.005$). There were small, but significant, effects of diet (CR greater than AL; $P < 0.05$) and genotype (KO greater than WT; $P < 0.01$) on basal 2DG uptake in the epitrochlearis (data not shown). There was a significant ($P < 0.001$) CR-induced increase in 2DG uptake in the presence of a high physiologic level (0.6 nmol/l) of insulin: 56 and 58% increase above basal in WT and KO mice, respectively. There was no effect of genotype. There was no statistically significant interaction between the effect of dietary treatment and genotype for 2DG uptake in EDL or epitrochlearis.

A sex difference was apparent for soleus 2DG uptake; therefore, the data for males and females were statistically analyzed and presented separately (Fig. 2). In male mice, soleus 2DG uptake in the absence of insulin was unaltered by dietary treatment, and was higher in KO compared with WT ($P < 0.01$). In the presence of a maximally effective dose of insulin (12 nmol/l), 2DG uptake by male soleus was significantly increased ($P < 0.001$) in CR relative to AL mice, and there was no effect of genotype. In female mice, there were significant main effects of dietary treatment (AL greater than CR; $P < 0.01$) and genotype (KO greater than WT; $P < 0.01$) on 2DG uptake by soleus (Fig. 2) in the absence of insulin (basal). Female soleus 2DG uptake in the presence of 12 nmol/l insulin was similar in all groups. In both male and female mice, there was no statistically significant interaction between the effects of dietary treatment and genotype on soleus 2DG uptake in the absence or presence of insulin.

Muscle IRS-1 and IRS-2 protein abundance. CR resulted in a significant decrease ($P < 0.05$) in IRS-1 protein abundance in gastrocnemius from WT mice (Fig. 3). As expected, IRS-1 protein was undetectable in KO mouse muscle. There was a significant main effect of genotype ($P < 0.05$, KO greater than WT) on IRS-2 protein abundance in gastrocnemius

TABLE 2
Plasma insulin, glucose, and leptin levels for mice homozygous for the intact (WT) and null (KO) IRS-1 alleles after 20 days of CR or AL feeding

	WT		KO		Main effects		Interaction
	AL	CR	AL	CR	D	G	D × G
Insulin (pmol/l)	98 ± 17	47 ± 0.9	248 ± 45	78 ± 27	<0.001	<0.005	NS
Glucose (mmol/l)	9.5 ± 0.7	7.1 ± 0.4	9.6 ± 0.7	6.7 ± 0.7	<0.001	NS	NS
Leptin (ng/ml)	6.9 ± 1.1	1.5 ± 0.5	4.0 ± 0.8	0.5 ± 0.3	<0.001	<0.05	NS

Data are means ± SE. For insulin and glucose, n = 11–13 per group; for leptin, n = 4–13 per group. Data analyzed by two-way ANOVA: D, main effect of dietary treatment; G, main effect of genotype; D × G, interaction between main effects. Leptin values are means ± SE for samples in which leptin was detectable. Analysis by nonparametric two-way ANOVA was performed on all data, with values below the detection limit of the assay assigned the same rank.

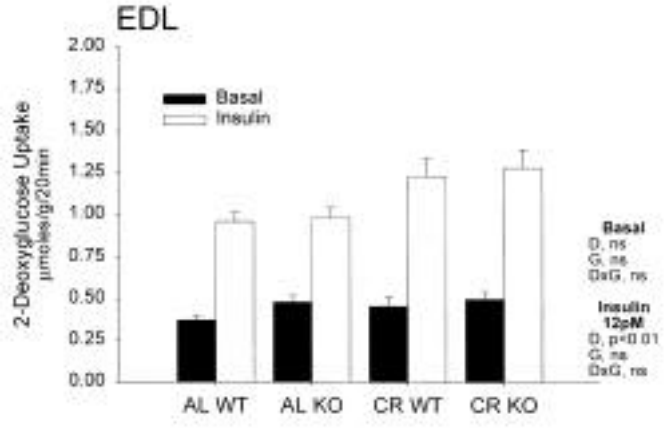

FIG. 1. Rate of 2DG uptake by EDL muscle from mice homozygous for the intact (WT) or null (KO) IRS-1 alleles after 20 days of CR or AL feeding. Rate of 2DG uptake was measured in the absence of insulin (■) and in the presence of 12 pmol/l insulin (□) and is expressed as micromoles 2DG per gram wet weight muscle per 20 min. Data are means ± SE for 11–13 mice per group. Data were analyzed by two-way ANOVA: D, main effect of dietary treatment; G, main effect of genotype; D × G, interaction between main effects.

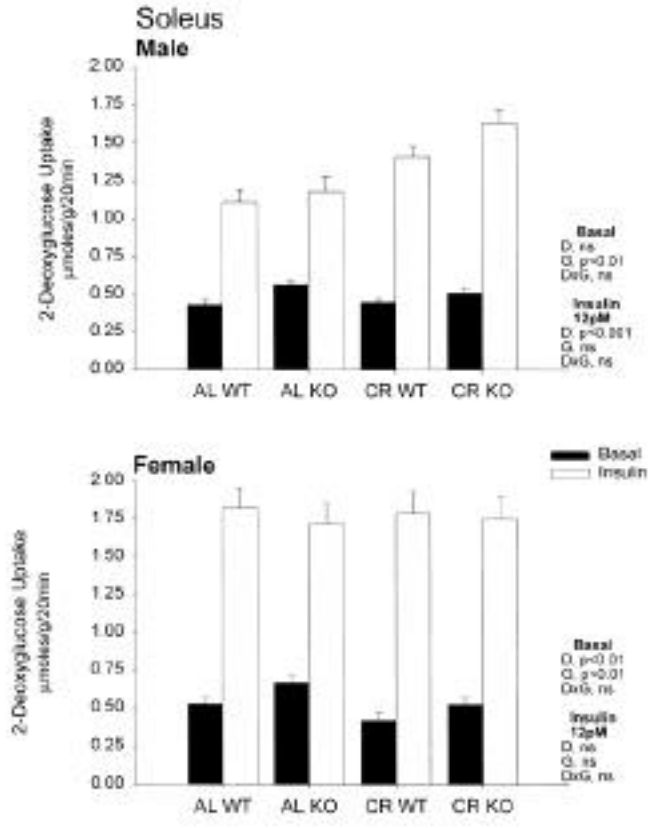

FIG. 2. Rate of 2DG uptake by soleus muscle from male and female mice homozygous for the intact (WT) or null (KO) IRS-1 alleles after 20 days of CR or AL feeding. Rate of 2DG uptake was measured in the absence of insulin (■) and in the presence of 12 pmol/l insulin (□) and is expressed as micromoles 2DG per gram wet weight muscle per 20 min. Data are means ± SE for 4–7 mice per group. Data within each sex analyzed separately by two-way ANOVA: D, main effect of dietary treatment; G, main effect of genotype; D × G, interaction between main effects.

muscle (Fig. 4), while dietary treatment had no effect. There was no significant interaction between main effects on IRS-2 protein abundance.

DISCUSSION

A moderate reduction in calorie intake (~20–40%) has well-known systemic consequences, including reductions in glycemia accompanied by a relatively greater decrease in insulinemia, enhanced insulin sensitivity, and weight loss with a disproportionate decrease in fat mass. These responses to CR have been documented in a number of species, including humans (3,10), rhesus monkeys (18), rats (19), and mice (20). The cellular defect(s) resulting in insulin resistance is largely believed to lie distal to insulin binding to its receptor. In addition to evidence that CR affects postreceptor steps in insulin-mediated glucose transport (13), we recently found that CR does not alter the timing or amount of insulin-stimulated increase in IRS-1-PI3K activity in muscle (14). This result suggested that CR might act by an IRS-1–independent mechanism, but it remained possible that CR alters some other aspect of IRS-1 function (e.g., by changing subcellular distribution of IRS-1-PI3K in muscle, or by an indirect effect that depends on extramuscular IRS-1 expression). Thus, the IRS-1 knockout mouse model was a valuable tool for investigating whether IRS-1 protein expression is essential for the insulin-sensitizing effects of CR. It was important to assess the impact of the absence of IRS-1 on the effects of CR at the level of an individual tissue (isolated skeletal muscle) and at the whole-body level (plasma insulin and glucose), because IRS-1 is expressed in many tissues (e.g., brain, adipose tissue, liver, kidney) (21).

The most important findings in this report are that IRS-1 protein expression is not essential for *1*) insulin-stimulated glucose transport by isolated skeletal muscles; *2*) the CR-induced increase in insulin-stimulated glucose transport by isolated skeletal muscles; or *3*) the CR-induced changes in glycemia, insulinemia, leptinemia, or loss of body fat mass. Thus, despite the total absence of IRS-1 protein in skeletal muscle isolated from mice null for the IRS-1 allele (KO), 2DG uptake in response to insulin is similar to muscle isolated from mice homozygous for the intact IRS-1 allele (WT), and the effect of 20-day CR on insulin-stimulated 2DG uptake is similar in KO and WT mice.

This study was apparently the first to evaluate the influence of CR on skeletal muscle glucose transport in mice. Insulin-stimulated glucose transport was increased in epitrochlearis and EDL, regardless of sex. Previous studies with rats have also found a CR-induced increase in insulin-stimulated glucose transport in epitrochlearis from males and females (11,14,15). Glucose transport in insulin-stimulated soleus was increased by CR compared with AL feeding for male, but not female mice. The reason for this sex difference in the soleus is uncertain.

The effect of 20-day CR on plasma insulin, glucose and leptin levels, and body weight and body fat was strikingly similar in KO and WT mice, indicating that IRS-1 expression is not essential for the systemic physiologic response to a dietary intervention that dramatically alters glucose homeostasis and insulin sensitivity, and suggesting that the effect of CR is mediated by an IRS-1–independent mechanism. The similar systemic response of KO and WT mice to CR is consistent with the similar in vitro 2DG uptake by muscle from KO and WT mice.

The mechanisms by which CR leads to enhanced insulin action in muscle are uncertain. In this study, fat mass was

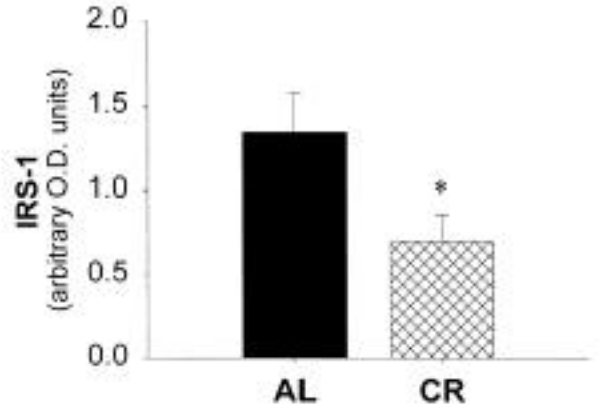

FIG. 3. IRS-1 protein abundance in gastrocnemius muscle from WT mice (homozygous for the intact IRS-1 alleles) after 20 days of CR (▨) or AL (■) feeding. IRS-1 levels are expressed as arbitrary units. Data are means ± SE for 11 mice per group. Data analyzed by *t* test. *$P < 0.05$, CR significantly different from AL.

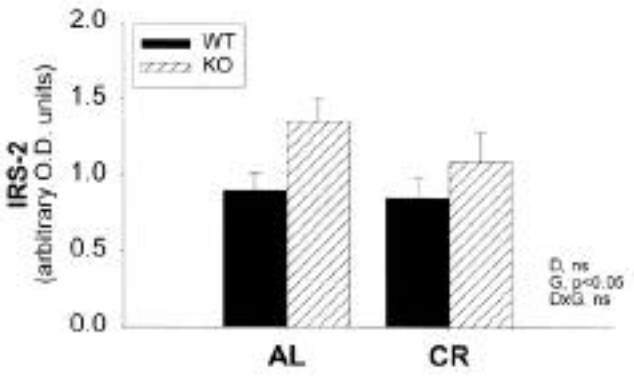

FIG. 4. IRS-2 protein abundance in gastrocnemius muscle from mice homozygous for the intact (WT) (■) or null (KO) (▨) IRS-1 alleles after 20 days of CR or AL feeding. IRS-2 levels are expressed as arbitrary units. Data are means ± SE for 8–11 mice per group. Data analyzed by two-way ANOVA: D, main effect of dietary treatment; G, main effect of genotype; D × G, interaction between main effects.

lower in CR compared with AL animals when muscle glucose transport was studied, but in previous studies, enhanced insulin sensitivity in muscle has been shown to occur after 4–5 days of CR (11,12), preceding detectable decreases in body fat (11). An attractive hypothesis is that changes in humoral factors (e.g., insulin, glucose, leptin) trigger the adaptations in skeletal muscle. CR resulted in an ~25–30% decrease in plasma glucose and an ~50–70% decrease in plasma insulin levels in both WT and KO mice. The magnitude of the effect of brief CR is similar to previous reports of long-term CR in mice (20). The effect of CR was similar in KO and WT mice, despite the fact that KO mice had consistently higher plasma insulin than WT mice. Thus, the effect of a brief period of moderate CR on plasma indices of whole body insulin sensitivity (glucose, insulin) was unaltered by the absence of IRS-1.

The findings reported here indicate that the cellular adaptations that result in the insulin-sensitizing effect of CR are intact in muscle from IRS-1 KO mice. The similar response of KO and WT muscle to CR may be accounted for by IRS-2. Although IRS-2 protein expression is not altered after 20-day CR in gastrocnemius from WT and KO mice, greater participation of IRS-2 in transducing the insulin signal to the GLUT4-enriched vesicles could still occur because of increased PI3K activity associated with IRS-2. In rats, we have also seen that 20-day CR results in a 50% decrease in muscle IRS-1 protein content and no change in IRS-2 abundance (14). However, the amount of IRS-1–associated PI3K activity is not changed in muscle from CR rats, indicating that a greater fraction of IRS-1 protein engages PI3K in CR animals. A similar increase in PI3K associated with IRS-2 may occur, and could explain the similar response to CR in WT and KO. Evidence exists that IRS-2 in isolated rat adipocytes can participate in GLUT4 translocation response to insulin (22). Insulin-stimulated IRS-2 phosphorylation is reportedly enhanced in muscle from IRS-1 KO relative to WT mice (23), and PI3K activity associated with IRS-2 is increased disproportionately more than the increase in tyrosine phosphorylation (23). An alternative explanation for the similar response of WT and KO to CR is that signaling elements distal to PI3K activation may be responsible for the increased GLUT4 translocation to the plasma membrane known to occur with CR.

The precise roles of IRS-1 and IRS-2 in the GLUT4 translocation response to insulin are not yet clear, and any compensatory alterations in the functional involvement of IRS-2 in insulin signaling in the absence of IRS-1 is highly speculative at this point. For example, in muscle from IRS-2 knockout mice, insulin-stimulated IRS-1 associated PI3K activity is decreased to <50% of that in WT mice (24). But, in IRS-1 knockout mice, insulin-stimulated PI3K activity associated with IRS-2 is increased more than twofold relative to WT mice (24). In addition, evidence suggests that subcellular trafficking of IRS-1 and IRS-2 with insulin stimulation is different (25). Also, association with PI3K differentially affects the tyrosine phosphorylation status of IRS-1 and IRS-2 (26). Thus, although IRS-1 and IRS-2 share similar functional primary amino acid sequences and secondary structural motifs, the specific participation of each IRS protein in insulin signaling is complex and remains undefined, even in intact (nonknockout) animals. Whether the small (~20 %) increase in muscle IRS-2 protein abundance in KO relative to WT mice observed in this study is functionally important is unknown.

There was no difference in the ability of muscle isolated from WT and KO mice to take up 2DG in response to insulin, and only in the soleus muscle was basal 2DG transport significantly different between KO and WT. The only other report of in vitro 2DG uptake in IRS-1 KO mice showed that 2DG uptake by soleus was 25% of that in WT mice in response to a maximally effective (100 nmol/l) dose of insulin (27). The reason for this difference in findings is uncertain, but may involve the specific genetic background of the different IRS-1 knockout lines. The mice in the report from Yamauchi et al. (27) were generated independently from the mice generated at Joslin; the mice used in the present report have a mixed genetic background of C57Bl6/129, and the genetic background of the mice generated by Yamauchi et al. is not described. Evidence that genetic background can be a determinant of skeletal muscle response to insulin has been previously reported in comparisons between inbred strains and F1 hybrid crosses of inbred strains (28). Another difference between the study by Yamauchi et al. and ours is the age of the animals. The fold-increase above basal for glucose transport in insulin-stimulated soleus muscles from male KO mice was very similar in our study (~2.1-fold) and the study by

Yamauchi et al. (~2.6-fold), suggesting that their KO mice were not more insulin-resistant than our KO animals. By contrast, their WT mice had a greater fold-increase in glucose transport with insulin treatment (~4.6-fold) compared with our WT animals (~2.6-fold). Yamauchi et al. studied 2- to 3-month-old mice and we studied 4.5- to 6-month-old animals. Perhaps, as in rats (29), age-related insulin resistance in skeletal muscle occurs between 2 and 6 months of age in WT mice, but not in KO mice, which are already relatively insulin resistant at 2–3 months of age.

The KO mice had smaller muscles than WT animals. However, for even the largest muscles from WT, the lag before complete equilibration of 2DG would represent a small fraction of the total incubation period (30), so the absence of differences in 2DG uptake by muscles from WT and KO mice is not an artifact of differences in muscle size. The 2DG transport data were analyzed for a correlation between muscle size and rate of 2DG transport. No consistent relationship was found between muscle size and rate of 2DG uptake (results not shown).

Consistent with earlier studies (16,31), our results indicate that the KO mice had moderate insulin resistance in vivo (i.e., hyperinsulinemia along with unaltered glycemia in KO compared with WT). In this context, the similar rates of insulin-stimulated in vitro glucose transport in WT and KO mice are unexpected. One possibility is that the in vivo difference between WT and KO mice is caused by an extramuscular factor, of humoral or neural origin, that is absent from the isolated muscle preparation. An obvious candidate is higher concentration of circulating lipids in IRS-1 KO animals. Lack of IRS-1 reportedly does not alter circulating FFA concentration, but plasma triglyceride concentration is ~60% higher in IRS-1 KO compared with WT mice (32), consistent with the association of chronic hyperinsulinemia and elevated circulating triglyceride-rich lipoproteins (33). Besides skeletal muscle, adipose tissue is a site for insulin-mediated glucose disposal, and insulin resistance in adipocytes isolated from IRS-1 KO mice has been demonstrated in vitro (16,31). This defect probably contributes to in vivo insulin resistance, but based on studies in rats (5), it seems unlikely that changes in glucose clearance by adipocytes could entirely account for the in vivo insulin resistance. Impaired insulin restraint of hepatic glucose output is another potential mechanism for in vivo insulin resistance. Regardless of the mechanisms accounting for the insulin resistance in IRS-1 KO animals, the CR-induced attenuation of in vivo insulin resistance in KO mice is likely attributable, at least in part, to the enhanced insulin action in skeletal muscle.

In conclusion, we have conducted a series of experiments in an attempt to identify the mechanisms underlying the improved insulin action that is characteristic of CR. We recently found no change in IRS-1 function (time course and magnitude of insulin-mediated IRS-1–associated PI3K activity) (14), suggesting that CR might act by an IRS-1–independent mechanism. Accordingly, in this study we used IRS-1–deficient mice to determine if IRS-1 is essential for the CR-induced increase in insulin action. The novel findings of this study demonstrate that IRS-1 protein is not required for the CR-induced increase in insulin-stimulated glucose transport in skeletal muscle, nor for insulin-stimulated glucose transport in muscle from AL mice. Furthermore, the absence of IRS-1 had no detectable bearing on any of the measured systemic adaptations to CR. Even in the complete absence of IRS-1, CR did not elicit greater skeletal muscle IRS-2 abundance, suggesting a diet-related influence on IRS-2 function (e.g., binding to PI3K), post-IRS aspects of insulin action, and/or IRS-independent events.

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