Brief Dietary Restriction Increases Skeletal Muscle Glucose Transport in Old Fischer 344 Rats

David J. Dean and Gregory D. Cartee

The primary purpose of this study was to determine the impact of brief dietary restriction (DR; 5 or 20 days) on skeletal muscle glucose transport activity (GTA) of 24-month-old female Fischer 344 rats. Basal GTA of isolated epitrochlearis muscles was unaffected by DR. Insulin-stimulated GTA was significantly increased by DR only at 20 days (+51%). We also assessed the influence of DR on energy sources (blood-borne and stored). An ~20% decline in glycemia occurred in each DR group, but plasma-free fatty acid and β-hydroxybutyrate concentrations were unaffected. Plasma insulin was reduced by 50% after 20 days. Hepatic glycogen was rapidly mobilized (~69% at 5 days; ~83% at 20 days). The depletion of visceral adipose stores was slower (no significant decline at 5 days; ~50% at 20 days), but the eventual reduction accounts for a significant amount of energy. The results demonstrate that muscle from old rats can rapidly upregulate GTA in response to brief DR. The relative magnitude of this increase represents a substantial portion of the increases previously observed after prolonged DR.

A PROLONGED period (i.e., months to years) of dietary restriction (DR) profoundly affects carbohydrate metabolism, increasing whole body insulin sensitivity for glucose metabolism in rats (Reaven et al., 1983), rhesus monkeys (Kemnitz et al., 1994), and humans (Wing et al., 1994). Skeletal muscle accounts for most of the insulin-stimulated blood glucose clearance (DeFronzo et al., 1981), so it is reasonable to suspect that adaptations in this tissue are important for glucose homeostasis. Consistent with this idea, restricting food intake for 19 months led to a 44% increase in glucose uptake in the perfused rat hindlimb (Ivy et al., 1991), in which skeletal muscle accounts for most of the glucose metabolism. By using the isolated epitrochlearis muscle preparation, we directly tested the hypothesis that skeletal muscle adapts to chronic DR. We found that DR, begun at ~3.5 months of age and continued thereafter, led to a >50% increase in insulin-stimulated, but not basal, glucose transport activity by muscles from rats across a wide range of the life span (i.e., 8–23 months) (Cartee et al., 1994). Such an extended period of reduced food consumption markedly diminishes body fat stores (Bertrand et al., 1980) that might, in turn, contribute to the observed improvements in muscle insulin sensitivity. More recently, we demonstrated that the DR-induced increase in insulin-stimulated glucose transport occurs in young adult rats (8 months old) after only 5 days of DR (Cartee and Dean, 1994). This rapid improvement in insulin action preceded a reduction in visceral fat stores. Furthermore, no additional enhancement in glucose transport was evident at 20 days of DR, despite the considerable reductions in visceral adipose mass that had occurred by that time. These findings do not refute the significance of body composition on whole body glucose regulation; rather, they demonstrate that the metabolic challenge of DR can rapidly induce adaptations intrinsic to the muscle that are independent of substantial changes in the amount of fat stored in visceral depots.

The purpose of the present study was to determine if the skeletal muscle from old rats retains the capability to rapidly upregulate glucose transport in response to brief DR. We studied the muscle in vitro, under highly controlled conditions, to isolate the DR-induced changes in the muscle itself from the changes in the prevailing extracellular environment that can indirectly influence muscle glucose metabolism. The intent is to eventually understand the impact of DR on energy metabolism by muscle, and other tissues, in the intact animal. As a preliminary step toward this long-range goal, we also assessed the influence of a brief period of DR on the levels of several important fuel reserves (liver glycogen, muscle glycogen, and visceral fat depots) and blood-borne substrates (glucose, free fatty acids, and β-hydroxybutyrate) during old age.

METHODS

Treatment of rats. — Female Fischer 344 (F344) rats were obtained from the National Institute on Aging colonies (Harlan Sprague Dawley, Indianapolis). In the Madison animal facility, rats were housed singly on a 12-hour light (0800–2000 hours) and 12-hour dark (2000–0800 hours) cycle, with temperature and relative humidity maintained between 23–24°C and 44–46%, respectively. After the animals had been in our facility for 5 days, daily food consumption (NIH-31, Teklad) was determined for 5 days to establish a baseline food intake. Food consumption was determined by weighing the food provided and correcting for the amount not eaten, including spillage. Animals were randomly assigned to one of three groups: ad libitum fed (AL), 20 days of dietary restriction (20DR), or 5 days of dietary restriction (5DR). At 0800 hour, the DR groups were provided with a food allotment that, based on baseline measurements, was expected to result in a food consumption equal to ~60% of ad libitum intake. Average food intake was determined 10, 5, and 1 day before the animals were killed. The rats were 24 months old at the time of the experiment.
Access to food was denied to all rats at 0800 hour on the day they were killed. The animals were weighed, and between 1000 and 1300 hours they were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight). The order of anesthetization was randomized, so that the mean time of tissue sampling did not vary among the groups. Blood was sampled from the tail vein for determination of glucose concentration, and the right and left epitrochlearis muscles were rapidly dissected out. Blood used for insulin, free fatty acid (FFA), and β-hydroxybutyrate determination was sampled via cardiac puncture, and the gastrocnemius, plantaris, and soleus muscles were dissected out and weighed. The following visceral fat pads were dissected out from the abdominal cavity and weighed: gonadal, consisting of the adipose tissue associated with the reproductive organs; perirenal, consisting of the adipose tissue associated with the kidneys; retroperitoneal, consisting of the adipose tissue associated with the dorsal wall of the peritoneal cavity; and omental-mesenteric, consisting of the adipose tissue associated with the digestive organs.

**Determination of blood glucose concentration.** — Blood glucose was determined by the glucose oxidase method with an ExacTech blood glucose sensor (Medisense, Cambridge, MA).

**Determination of plasma insulin concentration.** — Plasma insulin levels were quantified in duplicate using a radioimmunoassay with rat insulin as the standard (Linco, St. Louis).

**Determination of plasma β-hydroxybutyrate concentration.** — Plasma was analyzed for β-hydroxybutyrate concentration using a β-hydroxybutyrate dehydrogenase assay (Sigma, St. Louis).

**Determination of plasma FFA concentration.** — Plasma FFA concentration was determined using the microassay method described by Miles et al. (1983).

**Incubation of isolated epitrochlearis muscles.** — For all incubation steps, isolated epitrochlearis muscles were placed in 25-ml Erlenmeyer flasks containing Krebs-Henseleit buffer (KHB) (Krebs and Henseleit, 1932) including 0.1% bovine serum albumin (BSA) and further supplemented as described below. Flask were placed in temperature-controlled shaking H_2O baths and continuously gassed with 95% O_2-5% CO_2.

Both epitrochlearis muscles were dissected from rats, and each muscle initially was placed in Erlenmeyer flasks containing 3 ml KHB + 0.1% BSA supplemented with 2 mM sodium pyruvate and 6 mM mannitol; the flasks were shaken in an H_2O bath maintained at 30°C for 30 minutes. One muscle from each pair was incubated in 100 μU/ml insulin while the contralateral muscle was incubated in the absence of added insulin. After the initial incubation period, these muscles were transferred to a second flask for determination of 3-O-methylglucose (3-MG) accumulation, as described below.

**Determination of muscle 3-MG transport rate.** — The rate of 3-MG accumulation was determined essentially as previously described (Young et al., 1986). Briefly, muscles were incubated for 20 minutes with shaking at 30°C in flasks containing 2 ml KHB supplemented with .1% BSA, 8 mM [3H]-3-MG (437 μCi/mmol), 2 mM [14C]mannitol (8 μCi/ mmol), and the same insulin as the preceding step. Using epitrochlearis muscles of sizes similar to those employed in this study, we have confirmed that 3-MG accumulation is linear under these experimental conditions (unpublished observations).

At the conclusion of this incubation period, muscles were rapidly blotted on filter paper dampened with ice-cold incubation medium, trimmed, and frozen between aluminum clamps cooled to the temperature of liquid N_2. Muscles were stored at −70°C until subsequently weighed, homogenized in perchloric acid, and analyzed as previously described (Young et al., 1986).

**Determination of muscle glycogen concentration.** — Epitrochlearis muscle glycogen concentrations were determined using aliquots of the perchloric acid homogenate by the amyloglucosidase method (Passonneau and Lauderdale, 1974).

**Determination of liver glycogen concentration.** — Portions of gastrocnemius muscles from the AL and 20DR groups were homogenized in ice-cold phosphate-buffered saline using a Kontes Duall glass-glass tissue grinder. Protein concentration in the homogenates was determined using the spectrophotometric bicinchoninic acid assay (Smith et al., 1985).

**Statistical analysis.** — One-way analysis of variance (ANOVA) was performed, and Dunnett’s post hoc test was then used to determine if individual groups differed from the AL control group. Further analyses were executed on any of the data sets that had unequal variances: (1) data were transformed to log10 values, and ANOVA was then performed on the transformed data that had attained equal variances; and (2) if unequal variances persisted after data transformation, a Kruskal-Wallis nonparametric ANOVA was performed. In each case, the reanalysis produced results that were consistent with those from the initial ANOVA. Therefore, all results are expressed based on the original ANOVA and Dunnett’s post hoc test.

**RESULTS**

Food consumption was 11.9 ± 0.3 g/day in the AL group. The average daily food intake was reduced in DR groups (p < .001) to 6.2 ± 0.2 g/day (i.e., 52% of the AL values). Body weight tended to be lower after 5 days (~7%), and the reduction (~15%) achieved statistical significance (p < .01).
after 20 days of DR (Table 1). No significant reductions in weights were detected for any of the muscles studied (Table 1). Although every fat depot mass tended to be reduced at 5 days of DR, these losses failed to reach statistical significance. However, after 20 days of DR, significant reductions were detected in the weight of the retroperitoneal (37%), gonadal (28%), peritoneal (22%), and omental-mesenteric (33%) fat pads (Table 2). At this time, the sum of the fat pad masses had declined by 30% (p < .01) compared to only a 15% reduction in total body weight.

Blood glucose concentration was reduced (~17%) below AL values (p < .05) after 5 days and was maintained at this level at 20 days of DR (Table 3).

Plasma insulin concentration was not significantly reduced below AL values after 5 or 20 days of DR, although by 5 and 20 days of DR insulin was reduced by 31% and 50%, respectively (Table 3). The intraassay coefficient of variation was 5.2%.

No significant diet-induced changes in the β-hydroxybutyrate concentration were detected (Table 3), nor were any statistically significant differences in plasma FFA found (Table 3).

Basal 3-MG transport rate was unaltered after 5 or 20 days of DR (Figure 1). Glucose transport activity in the presence of 100 μU/ml insulin was not significantly altered after 5 days of DR, but a 51% increase (p < .05) was detected after 20 days.

Epitrochlearis glycogen concentration tended to be ~20% lower in each of the DR groups compared to the values for AL rats, but the difference did not achieve statistical significance (Table 4).

Hepatic glycogen concentration was reduced (p < .001) by 69% and 83% at 5 and 20 days of DR, respectively (Table 4).

No statistically significant difference in gastrocnemius protein concentration was observed (AL = 181.5 ± 5.0 mg protein/g muscle, DR20 = 172.2 ± 5.3).

DISCUSSION

The results demonstrate that skeletal muscle from old rats retains the capability to rapidly upregulate glucose transport in response to brief DR. As previously demonstrated for old rats with prolonged DR (Cartee et al., 1994) and for young rats with brief DR (Cartee and Dean, 1994), the adaptation was specific for insulin action, as no change in basal transport occurred. The relative magnitude of the increase observed in the old rats in this study (51%) after 20 days of DR is comparable to the changes previously observed in young rats (38%) after 20 days of DR (Cartee and Dean, 1994).

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**Table 1. Body Weights and Skeletal Muscle Weights**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Body Weight, g</th>
<th>Epirochlearis Weight, mg</th>
<th>Gastrocnemius Weight, mg</th>
<th>Plantaris Weight, mg</th>
<th>Soleus Weight, mg</th>
<th>Sum of Muscles Weight, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>294 ± 6</td>
<td>54 ± 2</td>
<td>952 ± 8</td>
<td>189 ± 5</td>
<td>85 ± 2</td>
<td>1280 ± 10</td>
</tr>
<tr>
<td>5DR</td>
<td>275 ± 6</td>
<td>49 ± 5</td>
<td>927 ± 35</td>
<td>190 ± 7</td>
<td>83 ± 3</td>
<td>1247 ± 45</td>
</tr>
<tr>
<td>20DR</td>
<td>251 ± 7*</td>
<td>57 ± 2</td>
<td>892 ± 23</td>
<td>190 ± 7</td>
<td>81 ± 4</td>
<td>1220 ± 33</td>
</tr>
</tbody>
</table>

*Significantly different from AL group (p < .01).

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**Table 2. Visceral Adipose Depot Weights**

<table>
<thead>
<tr>
<th>Group</th>
<th>Retroperitoneal Weight, g</th>
<th>Gonadal Weight, g</th>
<th>Perirenal Weight, g</th>
<th>Omental-Mesenteric Weight, g</th>
<th>Sum of Depots Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>4.69 ± 0.61</td>
<td>13.48 ± 0.95</td>
<td>2.37 ± 0.14</td>
<td>8.30 ± 0.78</td>
<td>28.83 ± 1.94</td>
</tr>
<tr>
<td>5DR</td>
<td>4.00 ± 0.38</td>
<td>13.29 ± 0.89</td>
<td>2.21 ± 0.15</td>
<td>7.99 ± 0.53</td>
<td>27.48 ± 1.71</td>
</tr>
<tr>
<td>20DR</td>
<td>2.94 ± 0.33*</td>
<td>9.70 ± 0.47†</td>
<td>1.85 ± 0.08*</td>
<td>5.57 ± 0.61†</td>
<td>20.07 ± 1.17†</td>
</tr>
</tbody>
</table>

*Significantly different from AL group (p < .05).

†Significantly different from AL group (p < .01).

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**Table 3. Blood Glucose, Plasma Insulin, Plasma β-Hydroxybutyrate, and Plasma FFA**

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glucose, mg/dl</th>
<th>Plasma Insulin, ng/ml</th>
<th>Plasma β-Hydroxybutyrate, mg/dl</th>
<th>Plasma FFA, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>103.7 ± 5.6</td>
<td>7.78 ± 1.9</td>
<td>3.70 ± 0.6</td>
<td>0.55 ± 0.036</td>
</tr>
<tr>
<td>5DR</td>
<td>86.1 ± 4.9*</td>
<td>5.33 ± 0.7</td>
<td>2.80 ± 0.5</td>
<td>0.50 ± 0.035</td>
</tr>
<tr>
<td>20DR</td>
<td>83.0 ± 3.6*</td>
<td>3.90 ± 0.7</td>
<td>3.58 ± 0.4</td>
<td>0.60 ± 0.067</td>
</tr>
</tbody>
</table>

*Significantly different from AL group (p < .05).
EFFECTS OF BRIEF DIETARY RESTRICTION

Figure 1. Effect of diet on 3-MG transport rate in rat epitrochlearis muscles incubated in the absence of insulin or with a submaximally effective insulin concentration (100 μU/ml). Values are means ± SE for 7–8 muscles/group. Results are expressed in μmol 3-MG accumulating • ml intracellular water•• • 20 min••••••. Open bars: muscles incubated in the absence of insulin; cross-hatched bars, muscles incubated in presence of 100 μU/ml insulin. AL, ad libitum fed rats; 5DR, 5 days of dietary restriction (DR); 20DR, 20 days of DR. *Significantly different from AL group (p < .05).

Table 4. Epitrochlearis Glycogen and Liver Glycogen

<table>
<thead>
<tr>
<th></th>
<th>Epitrochlearis.</th>
<th>Liver.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/g</td>
<td>μmol/g</td>
</tr>
<tr>
<td>AL</td>
<td>25.0 ± 1.8</td>
<td>330.3 ± 53.6</td>
</tr>
<tr>
<td>5DR</td>
<td>19.5 ± 1.3</td>
<td>101.7 ± 15.4*</td>
</tr>
<tr>
<td>20DR</td>
<td>19.6 ± 2.5</td>
<td>54.6 ± 8.1*</td>
</tr>
</tbody>
</table>

Note: Values are means ± SE for 6–8 rats per group. *Significantly different from AL group (p < .001).

Furthermore, the relative magnitude of this increase represents a substantial portion of the increases observed after a prolonged period of DR (i.e., 44–105% after ~5 to 24 months) (Reaven, et al., 1983; Ivy, et al., 1991; Friedman, et al., 1992; Cartee and Dean, 1994). Although the magnitude of the increase in glucose transport is apparently undiminished with advancing age, there is evidence suggesting a delayed onset for the older animals: studying younger (8-month-old) rats, we previously found that glucose transport activity was significantly increased after only 5 days of DR (Cartee and Dean, 1994).

GLUT-4, also known as the insulin regulatable glucose transporter, is the predominant glucose transporter protein in muscle (Klip and Paquet, 1990). In response to insulin, GLUT-4 transporters migrate from the intracellular compartment to the surface membranes (Klip and Paquet, 1990). It seems likely that the mechanism for enhancement of insulin-stimulated glucose transport involves the GLUT-4 protein, but we have found that prolonged DR does not increase the total abundance of GLUT-4 in muscle (Cartee et al., 1994). Presumably, DR enhances insulin-stimulated GLUT-4 translocation to the surface membranes and/or the flux rate of glucose through each transporter (i.e., increased intrinsic activity).

Blood glucose concentration was reduced after 5 days of DR, with essentially no further decrement after 20 days of DR. The time course and magnitude of the decline in blood glucose levels are comparable to the reduction we previously found for young rats after brief DR (Cartee and Dean, 1994). With prolonged DR, we have found greater reductions in blood glucose concentration (Cartee et al., 1994).

After 20 days of DR in this study, there was a trend for serum insulin to decline (i.e., 50% below AL values), but this apparent difference did not attain statistical significance. In adult and aged rats subjected to chronic DR, circulating insulin concentration decreased by ~40–70% (Masoro et al., 1982). We previously found a comparable decline (69%) in 8-month-old rats after only 20 days of DR (Cartee and Dean, 1994).

In light of the reduced blood glucose, we anticipated a possible compensatory increase in other blood-borne energy substrates. Supporting this conjecture, a similar dietary protocol (~34% reduction in food intake for 21 days) resulted in a 5-fold increase in circulating β-hydroxybutyrate concentration in rapidly growing, young rats (Yoo, et al., 1989). However, we found that DR had no effect on plasma β-hydroxybutyrate concentration in older rats. Although the interventions were very similar for the two studies, the metabolic challenge would likely be much greater for the rapidly growing rats. Chronic DR was found not to affect serum ketone levels, but in that study, food was removed from all animals 15 hours before collecting the blood samples (Masoro, et al., 1982).

The observation that plasma FFA levels were unaltered by DR indicates that FFA clearance kept pace with the accelerated release of endogenous FFA that would accompany the substantial loss in visceral adipose mass. Results from several earlier studies suggested that chronic DR also has little or no effect on circulating FFA levels in adult and aged mice (Harris, et al., 1994) and rats (Liepa, et al., 1980), except when an extended period of fasting is superimposed on the DR.

The body’s carbohydrate stores are quite limited, and their energetic contribution to the energy deficit of brief DR is small and transitory. After 20 days of DR, hepatic glycogen concentration had fallen to only 17% of the AL values. Assuming a liver mass of 7.5 g (Kanai, et al., 1985), the total net energetic loss from liver glycogen would be ~1.5 kcal. A large fraction of the decline in hepatic glycogen probably occurred during the initial 24 hours of DR (Lavoie, et al., 1987).

With the depletion of liver glycogen, hepatic glucose production is derived through gluconeogenesis, and amino acids derived from protein degradation serve as important gluconeogenic precursors. Skeletal muscle accounts for most of the body’s protein reserves, but there was no decrease in the mass of any of the muscles studied and gastrocnemius protein concentration was unaffected by DR, arguing against large losses in muscle protein content. Chronic DR, which does result in lower muscle mass, has also been shown not to reduce muscle protein concentration (Yu et al., 1982). Other lean tissues (e.g., liver, gastrointestinal tract) might have...
undergone net proteolysis, yielding amino acids that could be converted to glucose or directly oxidized. The glycerol originating from lipolysis could also support gluconeogenesis.

The small and statistically nonsignificant decline in muscle glycogen after 5 days of DR, along with no further decline after 20 days of DR, is reminiscent of the results of brief DR on younger rats (Cartee and Dean, 1994). Even after ~20 months of DR, muscle glycogen did not decline further in 23-month-old rats (Cartee et al., 1994). If the results for the epitrochlearis are representative of the remainder of the musculature, muscle glycogen stores are largely spared during brief DR.

The minor caloric contribution from carbohydrate supplies, along with the maintenance of muscle mass and protein concentration, implies a reliance on other energy depots. Visceral fat reserves were mobilized more slowly than glycogen (no detectable decline in fat pad mass was evident after 5 days of DR), but ultimately the adipose tissue provided a great deal more energy. Assuming that lipid accounts for 90% of adipose mass and the caloric density of lipid equals 9 kcal/g, the 8.76 g loss of visceral fat would have an energy equivalent of 71 kcal. Based on the oxygen consumption reported for 23-month-old female F344 rats (McDonald et al., 1989a), we estimate an energy expenditure of 31 kcal/day in our AL-fed rats. A transient decline (~20%) in metabolic rate has been noted during the first several weeks of DR (McCartee and McGee, 1989). Therefore, the energy expenditure for the DR rats would be predicted to be ~25–31 kcal/day (i.e., 500–620 kcal for 20 days). Taken together, these findings suggest that the reduction in visceral fat mass, if completely oxidized, could account for ~14% of the estimated energy expenditure of the DR rats. The total fat mass of 23-month-old female F344 rats equals 31.1% of body weight (McDonald et al., 1989b). Based on this value and the body weight of the AL-fed rats, the initial body fat content can be estimated to equal 91.4 g (i.e., 294 g x 0.311). If the relative decline in visceral fat mass (~30%) is representative of other lipid depots, then a total fat loss of 27.4 g would be predicted. This amount of fat has a caloric equivalent of 222 kcal, which corresponds to ~40% of the estimated energy expenditure.

In conclusion, the most important new observation of this study was that 24-month-old rats retain the capacity to rapidly upregulate insulin-stimulated glucose transport in response to a brief period of DR. The relative magnitude of this adaptation represents a substantial portion of the effect previously found after an extended period of DR. In addition, the DR-induced hypoglycemia was not accompanied by a reciprocal increase in plasma FFA and β-hydroxybutyrate concentrations. The results also demonstrate that DR leads to a rapid mobilization of hepatic glycogen, with only negligible further reduction after 5 days of DR. The onset for depletion of visceral adipose depots was slower, but the eventual decrease in these reserves represented a significant amount of energy.

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Address correspondence to Dr. Gregory D. Cartee, Biodynamics Laboratory, University of Wisconsin, 2000 Observatory Drive, Madison, WI 53706.

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