Lower calorie intake enhances muscle insulin action and reduces hexosamine levels

ANNIE C. GAZDAG, THOMAS J. WETTER, ROBERT T. DAVIDSON, KATHERINE A. ROBINSON, MARIA G. BUSE, ALICE J. YEE, LORRAINE P. TURCOTTE, AND GREGORY D. CARTEE

Biodynamics Laboratory, Departments of Nutritional Sciences and Kinesiology, University of Wisconsin, Madison, Wisconsin 53706; Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29425; and Department of Exercise Sciences, University of Southern California, Los Angeles, California 90089

Gazdag, Annie C., Thomas J. Wetter, Robert T. Davidson, Katherine A. Robinson, Maria G. Buse, Alice J. Yee, Lorraine P. Turcotte, and Gregory D. Carree. Lower calorie intake enhances muscle insulin action and reduces hexosamine levels. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R504–R512, 2000.—Previous studies have demonstrated enhanced insulin sensitivity in calorie-restricted CR, fed 60% ad libitum (AL) one time daily) compared with AL-fed rats. To evaluate the effects of reduced food intake, independent of temporal differences in consumption, we studied AL (unlimited food access)-fed and CR (fed one time daily) rats along with groups temporally matched for feeding [fed 3 meals (M) daily]: MAL and MCR, eating 100 and 60% of AL intake, respectively. Insulin-stimulated glucose transport by isolated muscle was increased in MCR and CR vs. AL and MAL; there was no significant difference for MCR vs. CR or MAL vs. AL. Intramuscular triglyceride concentration, which is inversely related to insulin sensitivity in some conditions, did not differ among groups. Muscle concentration of UDP-N-acetylhexosamines [end products of the hexosamine biosynthetic pathway (HBP)] was lower in MCR vs. MAL despite unaltered glutamine-fructose-6-phosphate aminotransferase activity (rate-limiting enzyme for HBP). These results indicate that the CR-induced increase in insulin-stimulated glucose transport in muscle is attributable to an altered amount, not timing, of food intake and is independent of lower triglyceride concentration. They further suggest that enhanced insulin action might involve changes in HBP.

hexosamine biosynthetic pathway; glutamine-fructose-6-phosphate amidotransferase; intramuscular triglyceride; insulin resistance; dietary restriction; meal feeding

INSULIN RESISTANCE for glucose utilization is a pathologic condition of increasing prevalence worldwide. Environmental factors such as physical activity and diet are known to influence whole body insulin sensitivity and glucose tolerance. Several dietary factors have been associated with changes in insulin resistance, including calorie intake. Chronic positive calorie balance results in obesity, a condition characterized by insulin resistance for glucose metabolism. Chronic moderate reduction in energy intake results in the opposite physiological condition, one of increased insulin sensitivity and improved glucose homeostasis. This insulin-sensitizing effect of moderate calorie restriction (CR) has been observed in several species, including humans, rhesus monkeys, and rats (6, 10, 21, 22, 29).

Improved whole body insulin sensitivity seen with CR is due in large part to increased skeletal muscle glucose utilization in response to insulin (10, 21). Long-term CR in rats (60% of ad libitum food intake) results in a 50–100% increase in insulin-stimulated glucose uptake by skeletal muscle relative to muscle from ad libitum (AL)-fed rats (7). The rate-limiting step for muscle glucose utilization appears to be glucose transport (40), and evidence suggests that glucose transport is the primary defect in impaired skeletal muscle glucose utilization seen in insulin resistance (17). Thus glucose transport represents a pivotal point in determining the muscle glucose metabolic response to insulin.

We have established a rodent model of brief (20 days) CR (6, 12) to study the cellular adaptations in muscle that account for the insulin-sensitizing effect of CR on glucose uptake. We and others have shown that a substantial portion of the increase in rat muscle insulin-stimulated glucose uptake after long-term CR (4–21 mo) is established within 4–20 days of CR (6, 10). This rapid adaptation is consistent with the time course for increased insulin sensitivity with reduced energy intake in humans (21).

Rats provided unlimited access to food will eat throughout the day (14). By contrast, rats limited to 60% of their AL intake typically eat their entire food allotment within 2–3 h of being fed (14); thus, for CR rats, a longer period of time would elapse between the last time of feeding and measurement of insulin action than for AL rats. It seemed possible that the increase in insulin-stimulated glucose transport that we observed in muscles from CR compared with AL-fed rats might be related to differences in timing, as well as amount, of food intake. The first aim of this study was to compare glucose transport in isolated muscles from AL-fed and CR rats that were matched for their pattern of food intake. Therefore, two groups of rats received their...
daily allotments of 100 or 60% of their AL intake in three meals. In addition, we compared these meal-fed groups with conventional AL (unrestricted access to food) and CR (60% of AL intake in a single daily allotment) rats. A reduction in calorie intake resulting in energy imbalance leads to a loss of body fat. Reduction in adipose mass has been clearly established with CR, but apparently no studies have evaluated the influence of CR on muscle triglyceride (TG) concentration. An inverse relationship between insulin-stimulated glucose uptake and intramuscular TG concentration has been observed in rats (36). A similar inverse relationship has been reported between muscle TG concentration and whole body insulin sensitivity in Pima Indians (26). The underlying reason for this frequently observed association is uncertain. It has been proposed that TG accumulation results in increased long-chain acyl-CoA concentration in the cytosol, which in turn might directly influence the activity of various enzymes of glucose metabolism or indirectly alter insulin signaling via diacylglycerol-induced activation of protein kinase C (23). Although a causal link between muscle TG concentration and insulin action has not yet been established, if muscle TG concentration is reduced with CR, the decrement might play a role in the increased insulin sensitivity with CR. Accordingly, a second major aim was to determine the influence of reduced calorie intake on skeletal muscle TG concentration.

Another putative mechanism for insulin resistance relates to a high rate of glucose flux through the hexosamine biosynthetic pathway (HBP; see Ref. 24). One factor that might influence this flux through HBP is the availability of glucose. A small decline in arterial glucose (~6%) in unaesthetized rats occurs with months of CR (40% lower food intake compared with AL controls; see Ref. 37), and a trend for lower glycemia (10%) was found after 4 days of CR (10). The metabolic consequences of this modest hypoglycemia are uncertain, but a greater change in glycemia in the opposite direction (hyperglycemia of ~50–100% above normoglycemia) can induce insulin resistance in perfused rat skeletal muscle (30). Furthermore, skeletal muscle insulin resistance of muscle strips dissected from persons with type 2 diabetes is reversed by in vitro exposure to normoglycemia (41). The HBP has been suggested as a possible mechanism for the reversible, hyperglycemia-induced insulin resistance. Glutamine-fructose-6-phosphate amidotransferase (GFAT), which catalyzes the conversion of fructose 6-phosphate (F-6-P) to glucosamine 6-phosphate (GlcN-6-P), is the rate-limiting enzyme for HBP. GlcN-6-P is rapidly converted to UDP-N-acetylhexosamines (UDP-HexNAc), which serve as essential substrates for glycosylation reactions. Several lines of evidence support the hypothesis that increased glucose flux through HBP mediates glucose-induced insulin resistance (18, 20, 31). Hexosamine-induced insulin resistance occurs independent of changes in GLUT-4 or insulin receptor (IR) expression, binding affinity, or tyrosine kinase activity, and it is secondary to diminished insulin-stimulated GLUT-4 translocation (2, 31). CR-induced increased insulin sensitivity is characterized by enhanced insulin-stimulated GLUT-4 translocation (12) despite unaltered GLUT-4 expression (7), IR number, binding affinity, or tyrosine kinase activity (1, 8). Thus an attractive hypothesis is that the CR-induced increase in insulin sensitivity is related to reduced flux through the HBP.

The third major aim of this study was to determine whether CR results in changes in skeletal muscle HBP by measuring GFAT activity and UDP-HexNAc concentration.

**METHODS**

Animal care and feeding. Male Fischer 344 (F344) rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) in two cohorts (cohort A, n = 50; cohort B, n = 48). Animals were housed singly in wire-bottomed cages under controlled environmental conditions (22–25°C, 40–50% relative humidity) with a 12:12-h light-dark cycle with lights on from 0600 to 1800. Animals were given ad libitum access to food (Teklad Rodent Diet No. 8604; Harlan Teklad, Madison, WI) and water. Rats were randomly assigned to one of four dietary treatment groups: AL, CR, meal "ad libitum" (MAL), and meal calorie restricted (MCR).

After a 2-wk acclimation period to the housing facility, baseline AL food intake was determined by measuring the amount of food given, the amount that spilled, and the amount that remained over several 2-day intervals. Average daily food consumption for rats was calculated and used as the basis for determining the amount of rat chow in each allotment of food given to CR, MAL, and MCR animals during the 20-day feeding protocol.

During the 20-day feeding period, AL-fed rats were allowed unlimited access to food. CR rats were given an allotment of food that corresponded to 60% of baseline AL intake. CR rats were given the full allotment of food at the start of the dark cycle (1800). MAL and MCR rats were given access to food for 1.5 h at three times: 1800, 2330, and 0600. The size of the meals was such that 40% of the total daily food allotment was provided at 1800 and 2330, and the remaining 20% of the daily allotment was given at 0600. The total amount of food given in the three meals was equal to either 100% (MAL) or 60% (MCR) of baseline AL food intake and spillage. For all rats, food intake and spillage were measured for days 1, 5, 10, 15, and 20 of the 20-day feeding period. When necessary, adjustments in food allotments were made to ensure that CR, MAL, and MCR animals consumed ~60, 100, and 60%, respectively, of their individual baseline AL intake.

On the day of the isolated muscle experiment, rats were transferred to the laboratory at 1000 and were kept in individual shoe-box cages. AL-fed animals were allowed access to food, and all animals had free access to water. On the day they were killed, animals (~7 mo old) were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) between 1400 and 1700. The order of anesthetization was randomized so that mean time of tissue sampling did not vary between groups. Once anesthetization was confirmed by loss of pedal reflex, epitrochlearis muscles were rapidly dissected out for in vitro incubation. Blood was drawn by cardiac puncture into EDTA-treated syringes. Plantaris muscle was immediately dissected out and frozen with tongs cooled to the temperature of liquid nitrogen. Whole liver (cohort A) was weighed and frozen, or a piece of liver (cohort B) was frozen with tongs as above. All tissues were stored at –80°C until analysis.

Retroperitoneal fat pads were carefully...
dissected from the abdominal wall and weighed. Epididymal fat pads (cohort A) were carefully dissected out and weighed. 3-O-methylglucosetran experiments. Immediately upon dissection from the animal, epitrochlearis muscles were placed in flasks of oxygenated Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 2 mM sodium pyruvate, and 6 mM mannitol and insulin (Humulin R; Lilly, Indianapolis, IN) at one of the following concentrations: none (both cohorts), 100 µU/ml insulin (cohort A), or 500 µU/ml insulin (cohort B). One muscle from each animal was used for determination of basal glucose uptake (no insulin), whereas the contralateral muscle was incubated with insulin. Flasks were gently agitated in a shaking water bath (30°C) and continuously gassed with 95% O2-5% CO2. After 30 min, muscles were transferred to flasks of KHB containing 8 mM [3H]3-O-methylglucose (3-MG; 0.25 mCi/mmol) and 2 mM [U-14C]mannitol (0.1 mCi/mmol; ARC, St. Louis, MO), and insulin levels were identical to those in the first incubation. After 15 min, muscles were blotted on filter paper moistened with ice-cold incubation media, rapidly trimmed of connective tissue, and frozen with aluminum tongs cooled to the temperature of liquid nitrogen. Muscles were stored at –80°C until further analysis.

Determination of 3-MG transport rate. Frozen muscles were rapidly weighed and homogenized in ice-cold 0.3 N perchloric acid (PCA) with glass-on-glass tubes (Kontes, Vineland, NJ). Uptake of 3-MG was determined as previously described (4) and is expressed as micromoles 3-MG per gram wet weight muscle per 15 min.

Plasma analysis. Collected blood was transferred to micro-centrifuge tubes and spun at 10,000 g for 10 min. Plasma was stored frozen at –20°C until analysis. Immunoreactive insulin was measured by RIA (Linco, St. Charles, MO). Glucose was determined by enzymatic (Trinder) assay (Sigma, St. Louis, MO). Leptin was assayed by ELISA (R&D Systems, Minneapolis, MN). Nonesterified free fatty acids (NEFA) were determined by colorimetric enzymatic assay (WACO, Richmond, VA).

Muscle and liver glycogen. Liver samples were hydrolyzed in 2 M HCl at 100°C for 2 h. Hydrolyzed samples were neutralized with 0.67 M NaOH and vortexed, and glycogen concentration was determined by the fluorometric amyloglucosidase method (27). Epitrochlearis glycogen was determined in PCA homogenates (27).

Muscle protein. Whole frozen plantaris muscles were weighed (cohort B), and a portion was homogenized in buffer (20 mM HEPES, 1 mM EDTA, and 0.25 M sucrose). Protein concentration was assessed by bicinchoninic acid assay (Sigma).

Intramuscular TG. Plantaris muscle (cohort B) TG concentration was determined as glycerol residues after extraction and separation of the muscle samples as previously described (15, 35). Briefly, lipids were extracted from powdered muscle samples by centrifugation in a chloroform-methanol solution (2:1, vol/vol) and 4 mM magnesium chloride. The organic extract was evaporated and reconstituted in chloroform, and silicic acid was added for the removal of phospholipids by centrifugation. The resulting supernatant was evaporated, saponified in ethanolic potassium hydroxide, and centrifuged with 0.15 M magnesium sulfate. The final supernatant was analyzed for glycerol spectrophotometrically by the enzymatic glycerol kinase method (Sigma).

Muscle nucleotide-linked hexoses and hexosamines. In plantaris from MAL and MCR (cohort A), UDP-HexNAc, UDP-Hex, GDP-mannose, and UDP levels were assessed as previously described (32). On the day of sampling, frozen muscles were powdered using a stainless steel mortar and pestle precooled to the temperature of liquid nitrogen, and the frozen powder was extracted as previously described (32). Extracted samples remained frozen at –70°C until analyzed (within 2 wk of processing) by HPLC as previously described (32). It was not feasible to rapidly prepare and analyze samples from all animals. Therefore, samples from the MAL and MCR groups in cohort A were analyzed. By studying these groups, the effect of amount of food intake, independent of temporal differences in food consumption, was assessed.

Muscle GFAT activity. GFAT activity in plantaris muscle was assayed as previously described (3, 32). Frozen muscles were stored at –70°C until assayed (within 24 h of sampling), and, as with hexosamine concentration, the analysis was limited to animals in the MAL and MCR groups (cohort B). Briefly, gel-filtered cytosolic extracts were incubated as described (3, 32) with 6 mM F-6-P and 12 mM glutamine. After 1 h, samples were deproteinized, and GlcN-6-P, the product of the GFAT-catalyzed reaction, was measured fluorometrically after derivatization with o-pthalaldehyde and HPLC separation. Data are expressed as picomoles GlcN-6-P generated per minute per microgram protein in the gel-filtered extracts (3, 32).

Statistical analysis. Data were analyzed by one-way ANOVA (Sigma Stat; SPSS, Chicago, IL), and Student-Newman-Keuls post hoc test was used to identify the source of significant variance. When only two groups of rats were compared, data were analyzed by two-tailed Student’s t-test.

RESULTS

Food intake. The pattern of food intake for both cohorts of animals was similar, and thus data were pooled. There was no difference in baseline food intake among the dietary treatment groups (AL, 15.6 ± 0.2; CR, 16.0 ± 0.2; MAL, 15.6 ± 0.2; and MCR, 15.8 ± 0.2 g/day). During the 20-day feeding period, AL rats maintained baseline levels (102.5 ± 1.3%) of food intake, and CR rats averaged 60.5 ± 0.6% of their individual baseline AL food intake. On the first day of feeding, food intake for the meal-fed animals was low (day 1: MAL 64.0 ± 3.2% and MCR 43.9 ± 2.3% of baseline intake), but animals rapidly adjusted feeding behavior so that they ate their full allotment of food during each 1.5-h meal. MCR began consuming their entire meals by the second meal of day 1. By day 3, MAL rats consumed on average 100.3 ± 1.2% of baseline AL intake.

Body weight. Body weights for both cohorts were similar, and thus data were pooled (Fig. 1). Baseline body weights were not different between cohorts in different dietary treatment groups. MAL rats lost weight initially; however, on the day of muscle incubation experiments, MAL had regained all lost weight. CR and MCR lost a similar amount of weight during the restriction period, losing 15.7 and 14.6%, respectively.

Muscle, liver, and adipose tissue mass. Plantaris muscle mass was not significantly different between dietary treatment groups (Table 1). Epitrochlearis mass was significantly lower (P < 0.05) in CR and MCR compared with AL and MAL. Liver mass was reduced after calorie restriction: CR and MCR were significantly (P < 0.001) smaller than AL and MAL. Epididymal (cohort A) and retroperitoneal (cohorts A and B) fat pad weights were significantly (P < 0.001) smaller in CR and MCR relative to AL-fed and MAL rats. These
was no difference in liver or fat pad weights between meal- and non-meal-fed animals matched for amount of food intake.

Epitrochlearis 3-MG uptake. Basal epitrochlearis 3-MG uptake in the absence of insulin was unaltered by dietary treatment (Fig. 2, A and B). With 100 µU/ml insulin, 3-MG uptake did not differ significantly among the dietary treatment groups. Insulin-stimulated 3-MG uptake above basal (calculated for paired muscles from each rat by subtracting 3-MG uptake without insulin from 3-MG uptake for the contralateral muscle incubated with insulin) with 100 µU/ml insulin tended to be higher (59–122%) for the CR groups compared with the AL-fed groups (Fig. 2A, inset), but the differences did not reach statistical significance (P = 0.148). When groups were pooled according to the amount of food eaten (AL + MAL and CR + MCR) and compared using a t-test, insulin-stimulated glucose transport with 100 µU/ml insulin was significantly higher (P < 0.05) for CR plus MCR compared with AL plus MAL.

Glucose transport activity with 500 µU/ml insulin for the CR group was significantly greater (P < 0.01) than the AL-fed or MAL groups (Fig. 2B). Glucose transport activity with 500 µU/ml insulin for the MCR group tended to be greater than the AL-fed and MAL groups (P = 0.06). Insulin-stimulated 3-MG uptake above basal (calculated as described above) in paired muscles used for 500 µU/ml insulin was significantly increased in CR (P < 0.01) and MCR (P < 0.05) relative to AL and MAL (Fig. 2B, inset). The CR and MCR values were 82–125% greater than the values for AL-fed and MAL groups.

Plasma analysis. Plasma leptin was significantly decreased (P < 0.001) in CR and MCR relative to AL and MAL (Table 2). Plasma NEFA were significantly higher (P < 0.05) in MAL relative to CR, and MCR. Plasma insulin and glucose values for both cohorts were pooled. Plasma insulin was significantly reduced in CR relative to AL, MCR (P < 0.05), and MAL (P < 0.001) and significantly higher in MAL relative to MCR (P < 0.001) and AL (P < 0.05). Plasma glucose levels were not significantly different among groups.

Muscle and liver glycogen levels. Glycogen concentrations in epitrochlearis muscle was similar among all dietary treatment groups: AL 35.9 ± 0.7; MAL 35.2 ± 1.0; CR 34.0 ± 1.4; and MCR 35.2 ± 1.1 µmol/g wet wt. Liver glycogen was lower in CR compared with all other groups (P < 0.05), and no significant difference was detected among the other groups: AL 190 ± 22; MAL 197 ± 23; CR 118 ± 12; and MCR 206 ± 12 µmol/g wet wt.

Muscle protein and TG content. Plantaris muscle protein content was similar in all dietary treatment groups: AL 184 ± 14.4; MAL 188 ± 11.8; CR 201 ± 11.5; and MCR 180 ± 7.2 mg protein/g wet wt muscle. Plantaris TG content was not significantly different between groups (Fig. 3).

Muscle nucleotide-linked hexose and hexosamines and GFAT activity. Plantaris muscle from MCR had significantly lower concentrations of UDP-HexNAc (P < 0.005), UDP-hexose (P < 0.05), and UDP (P < 0.05) than in muscle from MAL (Table 3). The ratio of UDP-HexNAc to UDP-hexose was significantly (P < 0.05) lower in muscle from MCR relative to MAL animals (0.852 ± 0.022 and 0.913 ± 0.013, respectively). GDP-mannose levels were not different between MAL and MCR. The activity of GFAT was not different in plantaris muscle from MCR and MAL rats (Table 3).

DISCUSSION

The most important new observations from this study are 1) a brief (20 day) reduction (40% below AL)

Table 1. Retroperitoneal and epidydimal fat pad and epitrochlearis, plantaris, and liver mass (g) after 20 days AL feeding, CR, MAL, or MCR

<table>
<thead>
<tr>
<th>Fat Pad</th>
<th>Retroperitoneal</th>
<th>Epidydimal</th>
<th>Plantaris</th>
<th>Epitrochlearis</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>2.72 ± 0.12</td>
<td>4.44 ± 0.27</td>
<td>0.266 ± 0.020</td>
<td>0.089 ± 0.002</td>
<td>12.1 ± 0.3</td>
</tr>
<tr>
<td>MAL</td>
<td>2.95 ± 0.15</td>
<td>4.56 ± 0.20</td>
<td>0.266 ± 0.025</td>
<td>0.087 ± 0.001</td>
<td>11.4 ± 0.3</td>
</tr>
<tr>
<td>CR</td>
<td>1.62 ± 0.12*</td>
<td>3.13 ± 0.16*</td>
<td>0.245 ± 0.025</td>
<td>0.080 ± 0.003†</td>
<td>8.6 ± 0.3*</td>
</tr>
<tr>
<td>MCR</td>
<td>1.44 ± 0.09*</td>
<td>2.96 ± 0.16*</td>
<td>0.256 ± 0.020</td>
<td>0.080 ± 0.002†</td>
<td>9.5 ± 0.3§</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 12 or 13 rats/group for epidydimal, plantaris, and liver, and n = 24–25 rats for retroperitoneal and epitrochlearis. AL, 20 days ad libitum feeding; MAL, meal feeding of 100% ad libitum intake; CR, 60% caloric restriction; MCR, meal feeding of 60% ad libitum intake. *P < 0.001, different from AL and MAL; †P < 0.05, different from AL and MAL; ‡P < 0.05, different from AL; §P < 0.05, different from CR by post hoc ANOVA.
in calorie intake can induce an increase in insulin-stimulated glucose transport by skeletal muscle independent of the pattern of feeding; 2) the CR-induced increase in insulin sensitivity occurs independent of a change in intramuscular TG concentration; and 3) reduced calorie intake can lead to decreased skeletal muscle UDP-HexNAc concentration, despite no change in maximal activity of GFAT, the rate-limiting enzyme of the HBP.

Earlier studies have demonstrated enhanced insulin-stimulated glucose transport in isolated skeletal muscle after 5–20 days of reduced (25–50% below AL) calorie intake. However, in these previous studies, CR rats provided their entire food in a single allotment have been compared with AL-fed rats given unlimited access to food (6, 12, 13). Rats provided unlimited access to food will eat throughout the day (14). CR rats limited to 60% of their AL intake typically eat their entire food allotment within 2–3 h of being fed (14), thus exhibiting “meal feeding” behavior as opposed to the nibbling feeding behavior of AL-fed rats. Therefore, differences in muscle glucose transport noted between the AL-fed and CR groups in earlier studies might have been related, at least in part, to differences in timing, as well as amount, of food intake.

Previous studies have demonstrated reduced body weight concomitant with increased insulin sensitivity when rats eating chow supplemented with cellulose were compared with chow-fed controls (11, 29). These results suggested that reduced calorie intake, despite unrestricted access to food, can lead to enhanced insulin action, but the effect of dietary cellulose, independent of reduced calorie intake, is uncertain, and the

Table 2. Insulin, glucose, leptin, and NEFA levels in plasma from rats fed AL, CR, MAL, or MCR for 20 days

<table>
<thead>
<tr>
<th></th>
<th>Insulin, ng/ml</th>
<th>Glucose, mg/dl</th>
<th>Leptin, ng/ml</th>
<th>NEFA, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>7.1 ± 0.6</td>
<td>239 ± 11</td>
<td>5.4 ± 0.5</td>
<td>155 ± 9</td>
</tr>
<tr>
<td>MAL</td>
<td>10.3 ± 1.0*</td>
<td>229 ± 8</td>
<td>6.0 ± 0.5</td>
<td>194 ± 14‡</td>
</tr>
<tr>
<td>CR</td>
<td>4.1 ± 0.3†</td>
<td>220 ± 8</td>
<td>2.5 ± 0.4§</td>
<td>145 ± 11</td>
</tr>
<tr>
<td>MCR</td>
<td>6.6 ± 0.7</td>
<td>239 ± 8</td>
<td>2.5 ± 0.4</td>
<td>136 ± 10</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 24 or 25 rats/group for insulin and glucose, and n = 12 or 13 for leptin and free fatty acids. NEFA, nonesterified fatty acid. *P < 0.005, different from AL, CR, and MCR; †P < 0.05, different from AL and MCR; §P < 0.001, different from AL and MAL by Student-Newman-Keuls post hoc ANOVA.

Fig. 2. Rate of 3-O-methylglucose (3-MG) uptake by epitrochlearis muscle from rats after 20 days of AL feeding, CR, MAL, or MCR. 3-MG uptake in the absence of insulin (hatched bars) and in the presence of insulin (hatched bars; 100 μU/ml in A and 500 μU/ml insulin in B) is expressed as μmol 3-MG·g wet wt muscle⁻¹·15-min incubation⁻¹. Insulin-stimulated 3-MG uptake (inset) was calculated by subtracting basal 3-MG uptake from 3-MG uptake in the presence of insulin. Data are means ± SE; n = 12 or 13 rats/group. *P < 0.01, CR different from AL and MAL and †P < 0.05, CR and MCR different from AL and MAL by Student-Newman-Keuls post hoc ANOVA.

Fig. 3. Intramuscular triglyceride concentration in plantaris muscle from rats after 20 days of AL feeding, CR, MAL, or MCR. Data are means ± SE; n = 12 rats/group.
magnitude of the reduction in calorie intake was not reported. Recently, Cusin et al. (10) studied CR rats (40% of AL intake for 4 days) that were fed two times daily (1/3 at 0600 and 2/3 at 1800) and AL-fed animals that were allowed unlimited access to food until 5 h before a euglycemic-hyperinsulinemic clamp, after which food was removed. They reported ~40–65% increases in the glucose-utilization index in skeletal muscles during the clamp. This approach lessened, but did not eliminate, changes in the temporal pattern of food intake between AL-fed and CR groups.

We extended the results from these studies by matching the feeding times for two groups of rats so that differences between the MAL and MCR groups were solely attributable to a 40% difference in food intake. Insulin-stimulated glucose transport was increased by ~100% in MCR compared with MAL groups, demonstrating enhanced insulin action independent of differences in timing of food intake. Furthermore, the pattern of food intake, at least for the feeding protocols we studied, did not significantly affect glucose uptake by muscle from animals matched for amount of daily food consumption. Insulin-stimulated 3-MG uptake was not different in epitrochlearis from AL and MCR, nor between muscles from CR and MCR, despite markedly different patterns of food intake.

Epitrochlearis glucose uptake in response to insulin in male F344 rats was quite low compared with our results in previous studies in which we studied female F344 (6, 13) or male hybrid (Brown Norway × F344, F1) rats (4, 5). The underlying cause of the insulin resistance in male F344 rats is uncertain, but here we report enhanced insulin-stimulated glucose transport with 500 µU/ml (and with 100 µU/ml for CR + MCR vs. AL + MAL), and we previously found significant increases in insulin-stimulated glucose transport in muscles from CR compared with AL-fed male F344 rats with 200 and 20,000 µU/ml (12). Taken together, these results demonstrate that CR enhances insulin-stimulated glucose transport in insulin-resistant, male F344 rats across a wide range of insulin concentrations.

Dietary intake of each macronutrient was 40% lower in CR and MCR groups compared with the AL-fed groups. We measured skeletal muscle energy stores (glycogen, TG, and protein) because, under some conditions, the amounts of lipid and carbohydrate accumulate by muscle are inversely related to insulin sensitivity. Increased intramuscular TG levels can be negatively correlated with insulin-stimulated glucose uptake (36). It seemed reasonable to suspect that a reduction in calorie intake might lead to reduced intramuscular TG concentration. However, intramuscular TG concentration was unrelated to calorie intake, demonstrating that the enhanced insulin action after brief CR is independent of depleted muscle TG stores. Of course, it remains possible that lipid composition of muscle membranes differed among the dietary groups and that CR for longer than 20 days might influence TG concentration. It is also conceivable that, despite no decline in muscle TG concentration, CR might influence insulin action via changes in other aspects of lipid metabolism (e.g., cytosolic long-chain acyl-CoA concentration). Reduced muscle glycogen concentration, for example after exercise, is associated with enhanced insulin-stimulated glucose transport. The lack of any CR-induced decline in muscle glycogen confirms findings in other studies of brief (12) and long-term (37) CR and demonstrates that the effect of CR on insulin action is not mediated by muscle glycogen depletion. There was also no significant difference in muscle protein concentration among the dietary groups, and muscle mass was only slightly lower (~4–10%) in rats with reduced calorie intake. We have previously demonstrated that the increased insulin-stimulated glucose transport with brief CR precedes any detectable change in muscle mass (6, 13). With long-term (months) CR (40% below AL), muscle mass is typically ~25–30% lower than AL controls (37), but protein concentration remains unchanged (25).

Despite no change in muscle glycogen concentration, glucose flux through individual metabolic pathways is altered by reduced calorie intake. We previously observed that, under in vivo conditions (with lower glycemia and insulinemia in CR vs. AL-fed rats), glucose-6-phosphate and F-6-P concentrations were lower in muscles from CR compared with AL-fed rats (37). F-6-P is a substrate for GFAT, the initial and rate-limiting enzyme for the HBP (24). The product of GFAT activity, GlcN-6-P, is readily N-acetylated and further metabolized to UDP-N-acetylglucosamine and UDP-N-acetyl-galactosamine, collectively UDP-HexNAc. UDP-HexNAc, the end products of HBP, are essential precursors for the synthesis of glycoproteins and proteoglycans (24, 32).

Several lines of evidence support the hypothesis that end products of HBP may play a role in modulating insulin’s action on glucose metabolism in skeletal muscle. GlcN is transported into the cell like glucose, albeit with lower affinity. On entry, GlcN is phosphorylated by hexokinase and thus enters the HBP, bypassing GFAT. Preexposure to GlcN impairs insulin-stimulated glucose transport in rat skeletal muscle in vitro (31) and in vivo (2) and impairs GLUT-4 translocation to the cell surface. In vivo, the effects of GlcN and of hyperglycemia are not additive, suggesting that both may induce insulin resistance by a common mechanism (34). Increased UDP-HexNAc concentrations in muscle correlate with whole body insulin resistance in re-
response to various in vivo treatments, e.g., sustained hyperglycemia or increasing plasma levels of GlcN, uridine, or free fatty acid (18, 19). Transgenic mice overexpressing GFAT in skeletal muscle and fat tissue develop insulin resistance (20). GlcN-induced insulin resistance in muscle develops without changes in GLUT-4 or changes in the expression, binding affinity, or tyrosine kinase activity of the IR (2, 31). Increased insulin sensitivity in muscles of CR rats reflects enhanced insulin-stimulated GLUT-4 translocation to the cell surface (12) and is also associated with unaltered total GLUT-4 protein (7) and IR expression, IR binding affinity, or tyrosine kinase activity (1, 8).

The concentration of UDP-HexNAc was significantly reduced in MCR relative to MAL, even though muscle activity of GFAT was similar in MAL and MCR. Based on the apparent Michaelis constant values of GFAT and cellular concentrations of its substrates (glutamine and F-6-P), flux through HBP is likely dependent on the availability of F-6-P (32). Consistent with this idea, muscle F-6-P levels are reduced in CR (37). The CR-induced decline in F-6-P and UDP-HexNAc suggests decreased glucose flux through HBP.

Regardless of the mechanism for the lower UDP-HexNAc concentration in muscles from CR rats, this reduction concomitant with increased insulin sensitivity is consistent with previous reports in vivo in which the converse was observed, i.e., increased muscle UDP-HexNAc levels were associated with insulin resistance of glucose transport/phosphorylation (3, 18–20, 31, 34). The processes linking the HBP to insulin action are uncertain, but UDP-HexNAc are known to be essential precursors for synthesis of glycoproteins, glycolipids, and proteoglycans (24, 32). Protein glycosylation plays an important role in posttranslational protein folding, stability, sorting, and function of proteins. GlcN infusion resulting in decreased insulin-mediated glucose clearance also leads to increased protein glycosylation in muscle (18, 39). The impairment in GLUT-4 vesicle translocation seen in hexosamine-induced insulin resistance (2) may be due to altered glycosylation of proteins involved in the insulin signaling or glucose transport systems. Consistent with this idea, GlcN infusion was recently demonstrated to result in increased O-linked N-acetylgalactosamine modification of several insulin-signaling proteins in muscle, including IR substrate (IRS)-1 and IRS-2, concomitant with reduced insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-1-associated phosphatidylinositol 3-kinase activity (28).

In this context, one speculative scenario would be that the CR-induced decline in UDP-HexNAc that we observed might be accompanied by a decline in glycosylation of key proteins, leading to alterations in their function and enhanced insulin action.

Glycemia did not differ significantly among the groups, but it tended to be lower in the CR compared with the AL-fed group. Blood was sampled by cardiac puncture after anesthesia with pentobarbital sodium, which can increase circulating glucose in rats (16). Thus absolute glucose values should be interpreted without caution, but the relative difference (8%) between AL-fed and CR rats compares with the relative reduction (4–10%) previously reported for arterial blood from unanesthetized CR vs. AL-fed rats (10, 37). We observed no evidence of hypoglycemia in the MCR rats, a finding supported by the lack of decline in liver glycogen concentration in this group. Taken together with data from earlier research, our results indicate that the CR-induced increase in insulin-stimulated glucose transport or the CR-induced decrease in UDP-HexNAc does not require extreme hypoglycemia.

Plasma insulin was affected by both calorie intake and pattern of food intake. The relative decline in insulin for CR vs. AL-fed rats (42%) compares with the 40–75% decline previously reported in unanesthetized rats (10, 37). Plasma insulin for MCR rats was significantly lower (36%) than for MAL animals, but the MCR group was not significantly different from the AL-fed group. Insulin levels were higher in meal-fed animals relative to the non-meal-fed animals consuming the same amount of food (MAL > AL, MCR > CR). Previous studies have provided conflicting results regarding whole body insulin action and glucose tolerance in rats that consumed all of their food in a few meals compared with AL controls with the same daily food intake (9, 33, 38).

We conclude from the data reported here that the increase in skeletal muscle insulin sensitivity for glucose metabolism seen after a brief period of reduced calorie intake is related to the reduction in calories consumed, independent of differences in timing of food intake. In addition, the influence of brief CR on insulin action is not attributable to changes in muscle energy stores, as evidenced by no changes in muscle concentrations of TG, glycogen, or protein. Finally, calorie restriction reduces muscle UDP-HexNAc concentration without altering GFAT activity. Assuming that UDP-HexNAc is an indicator of glucose flux via HBP, one may speculate that reduced glucose flux through this pathway may contribute to the enhanced insulin sensitivity of glucose metabolism with CR.

Perspectives

Many studies have addressed the link between energy balance and insulin action, although usually from the perspective of positive rather than negative calorie balance. Understanding the mechanisms underlying the CR-induced increase in insulin action is valuable because 1) the link between energy balance and insulin action is a fundamental biological phenomenon found in many species, and 2) in addition to enhanced insulin action, moderate CR without malnutrition can provide many other beneficial outcomes with few negative consequences. The practical problem in treating human obesity, insulin resistance, and type 2 diabetes is the inability to maintain this lifestyle change over a long duration in free-living conditions. Clinical success might be better achieved if a drug that replicates the benefits of CR could be developed. Toward this aim, identification of the molecular signals that couple whole...
body calorie intake with muscle glucose transport would be quite useful. Whether changes in the HBP, as evidenced by the CR-induced decline in UDP-HexNAC, have any causal role for enhanced insulin action remains to be determined. Nonetheless, the present findings, taken together with evidence from earlier studies, make this possibility worth further investigation.

We acknowledge the assistance of Mark Obermyer, Charles L. Dumke, Kenneth Fedner, and Erin Maslowski.

This study was supported by National Institutes of Health Grants R01-AG-10026 (to G. D. Cartee), T32-AG-00213 (to A. C. Gazdag), and R01-DK-02001 (to M. G. Buse).

Address for reprint requests and other correspondence: G. D. Cartee, Biodynamics Laboratory, Univ. of Wisconsin, 5000 Observatory Dr., Madison, WI 53706 (E-mail: cartee@education.wisc.edu).

Received 14 June 1999; accepted in final form 17 September 1999.

REFERENCES


