Effect of calorie restriction on in vivo glucose metabolism by individual tissues in rats

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Wetter, Thomas J., Annie C. Gazdag, David J. Dean, and Gregory D. CarTEE. Effect of calorie restriction on in vivo glucose metabolism by individual tissues in rats. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E728–E738, 1999.—We evaluated the effects of 8 mo of calorie restriction [CR: 60% of ad libitum (AL) food intake] on glucose uptake by 14 tissues in unanesthetized, adult (12 mo) F344×BN rats. Glucose metabolism was assessed by the 2-[3H]deoxyglucose tracer technique at 1500 or 2100. Despite an ~60% decline in insulinemia with CR, plasma 2-[3H]deoxyglucose clearance for CR was greater than for AL at both times. A small, CR-related decrease in glucose metabolic index (Rg) occurred only at 1500 in the spleen and heart, and this decrease was reversed at 2100. In some tissues (cerebellum, lung, kidney, soleus, and diaphragm), Rg was unaffected by diet, regardless of time. In the other tissues (brown fat, 3 white fat pads, epitrochlearis, plantaris, and gastrocnemius), Rg was higher or tended to be higher for CR vs. AL at one or both times. These findings indicate that 8 mo of CR did not cause a continuous reduction in in vivo glucose uptake by any tissue studied, and, in several insulin-sensitive tissues, glucose uptake was at times greater for CR vs. AL rats.

GLUCOSE HOMEOSTASIS is altered by a moderate reduction in calorie intake (~25–40% below ad libitum (AL) values). Clarification of the nature of this relationship has important implications. Calorie restriction (CR) is a common treatment for obesity, insulin resistance, and type 2 diabetes (1). CR has also elicited a great deal of interest among gerontologists because in a variety of species, including rats, CR extends maximal life span and delays many age-related changes in a number of different tissues (e.g., skeletal muscle, myocardium, brain, liver, spleen, lung, and kidneys; see Ref. 42). It has been suggested that some of the consequences of CR might be attributable to altered glucose metabolism (32, 33). Although CR can lower glycemia and insulinemia, these parameters are not the only determinants for tissue glucose uptake and metabolism. In fact, CR enhances insulin-stimulated glucose transport and/or metabolism by in vitro preparations of skeletal muscle (4) and adipocytes (5), so it seemed possible that CR might result in unchanged or even increased in vivo glucose uptake by these tissues. Little is known about the influence of CR on glucose metabolism by other tissues. For example, the brain depends almost exclusively on glucose for energy under most conditions and is highly susceptible to CR-induced alterations (14), so it would be of great interest to determine if CR alters its in vivo glucose uptake. No previous study has evaluated in vivo glucose uptake by individual tissues in unanesthetized rats undergoing prolonged, moderate CR.

Therefore, the purpose of this experiment was to determine if CR alters in vivo glucose flux into a number of tissues, including several skeletal muscles, white adipose tissues, brown adipose tissue (BAT), heart, brain, kidney, spleen, and lung. Earlier studies have offered valuable insights by comparing AL and CR animals using the glucose clamp procedure or glucose tolerance tests (26, 39, 41). However, unlike these earlier studies, our goal was to compare AL and CR animals under their usual living conditions (unanesthetized and unrestrained) with ambient glucose and insulin. Toward that end, we used the 2-deoxyglucose (2-DG) tracer method to determine the glucose metabolic index (Rg) of individual tissues (12, 28, 43). We compared AL-fed rats, with unlimited access to food, with rats that were subjected to a CR regimen that is used in the rodent colonies supported by the National Institute on Aging (i.e., limiting caloric intake to ~60% of AL consumption with the food provided at the beginning of the dark cycle). This protocol has been used by many investigators to demonstrate profound adaptations to CR.

When compared with AL animals, rats fed according to this CR procedure rely relatively more on lipid oxidation during the latter portion of the light cycle and relatively more on carbohydrate oxidation during the early portion of the dark cycle (35). Therefore, we studied animals during the light and dark cycle. To gain further insight into the influence of CR on glucose metabolism, we also assessed circulating levels of glucose, insulin, C-peptide, free fatty acids (FFA), and lactate and the concentrations of glycogen, glucose 6-phosphate (G-6-P), and fructose 6-phosphate (F-6-P) in skeletal muscle and liver. We hypothesized that plasma 2-DG clearance would be faster for AL compared with CR rats during the light cycle (concomitant with the greater reliance on carbohydrate oxidation by AL rats), and the reverse would be true during the dark cycle (when carbohydrate oxidation is greater in CR rats). We further hypothesized that these diurnal differences would be attributable in large part to diet-related changes in Rg of skeletal muscle, the tissue that accounts for most of the insulin-sensitive glucose clearance (10).

METHODS

Treatment of rats. Male Fischer 344 × Brown Norway (F344×BNF1) rats, aged 11–12 mo, were obtained from the
National Institute on Aging colony [National Center for Toxicology Research (NCTR) J efferson, AR]. One-half of the animals (n = 20) were AL fed, and the other one-half (n = 20) was on a CR diet (caloric intake had been limited to ~60% of AL from 16 wk of age); assignment to the diet groups was random. Housing conditions in our animal facility corresponded to those at the NCTR: 12:12-h light-dark cycle with lights on from 0600 to 1800; temperature 23 ± 0.6°C; relative humidity = 50 ± 5%; individual housing in polycarbonate shoe box cages with stainless steel wire lids and wood chip bedding. AL rats had free access to water and rat chow (NIH-31, Mix 5022; PMI Feeds, St. Louis, MO). CR rats had free access to water and, at 1800, were provided food equal to ~60% of the intake of AL rats using an NIH-31 diet supplemented with sufficient vitamins so that the AL and CR group vitamin intakes were essentially equal (Mix 5L38; PMI Feeds). Food consumption was determined by weighing the food provided and correcting for food not eaten.

Surgical procedure for cannulating the carotid artery. After at least 2 wk of acclimation to the Madison facility, rats were anesthetized (between 0800 and 1200) by an intraperitoneal injection of a cocktail including ketamine (60 mg/kg; Ketaject, Phoenix Pharmaceutical, St. Joseph, MO), xylazine (3.2 mg/kg; Xyla-ject, Phoenix Pharmaceutical), and acepromazine (0.4 mg/kg; Fermitamina, Kansas City, MO). Under sterile conditions, a small incision was made in the left carotid artery, and a catheter (2 cm of PE-10 tubing inserted and glued into PE-50 tubing) was introduced and advanced ~2 cm to the aortic arch (verified at autopsy). The catheter was secured using silk suture (5–0). The catheter was exteriorized between the scapulae, and nonirritating Silastic tubing (Baxter Healthcare, Deerfield, IL) was placed over the exterior tip of the catheter, advanced to the outer surface of the exit site, and glued in place to prevent the catheter from slipping under the skin. The catheter, flushed with sterile saline and cut so that ~2 cm were exteriorized, was stopped using 25-gauge stainless steel wire (Small Parts, Miami Lakes, FL). The incisions on the neck and between the scapulae were closed with silk suture (4–0).

Post surgery body weight and food consumption were determined at 1800 on the day of surgery (day 0), the following day (day 1), and every other day thereafter (i.e., days 3, 5, 7, etc.). Food consumption for days 0 and 1 represented the amount eaten during the previous 24 h, whereas subsequent food consumption was determined for 48-h blocks of time.

Determination of tissue 2-DG uptake in vivo. Rats from each diet group (AL and CR) were randomly assigned to the 1500 or the 2100 group. These designations refer to the time at which radiolabeled 2-DG was injected into the animals. As the CR rats were fed at 1800, these times correspond to 3 h before (1500) or 3 h after (2100) the feeding of CR rats. It was important to minimize the possible influence of surgery-related reductions in body weight or food intake. Therefore, all rats were allowed at least 12 days of recovery after catheter placement, and the 2-DG injection procedure was not performed until body weight and food intake approximated baseline values (~95–100%) for ≥7 days. Approximately 30 min before 2-DG injection, the catheter was lengthened with PE-50 tubing so that sampling could be performed without disturbing the animal. The catheter extension did not restrict the animal’s movement. The animals remained in their home cages while 2-DG was injected; access to food and water remained unaltered. For the 2100 measurements, the room lights remained off, and a small reading light (shielded from the animal) provided low light to enable blood sampling. At the appropriate time, 2-[3H]DG (~30 µCi; New England Nuclear, Boston, MA), which had been evaporated and reconstituted in sterile saline to eliminate any contaminating H2O, was injected as a bolus into the carotid catheter. The catheter was immediately filled and flushed with blood three times, followed by a flush with sterile saline (500 µl) to remove residual radioactivity. If the catheter was not patent on the day planned for the experiment, the catheter was filled with urokinase (Abbokinase Open-Cath; Abbott Laboratories, North Chicago, IL). If patency was reestablished ~10 min later, the experiment proceeded as planned. Otherwise, urokinase was left in the catheter, and patency was again tested on a subsequent day. If the catheter was patent at this time, the experiment was performed. If not, the animal was eliminated from the study.

Blood (~200 µl) was sampled from the same catheter immediately before injection of 2-[3H]DG (0 min) and at 1, 3, 5, 10, 15, and 25 min after tracer injection; at 45 min, 1 ml of blood was sampled. After all but the final blood sample, the catheter was flushed with an equivalent volume of sterile saline. Blood was drawn into syringes containing anticoagulant and was transferred to microfuge tubes on ice until they were centrifuged. The resultant plasma was stored at ~80°C.

Immediately after the 45-min blood sample was taken, pentobarbital sodium (60 mg/kg) was injected into the catheter, and animals were rapidly anesthetized. Rats were immediately moved to an adjacent room, and the following tissues were rapidly dissected out: liver (left lateral lobe), spleen, soleus, plantaris, gastrocnemius, epimysitic, kidney, diaphragm, heart (both ventricles), lungs, brain (cerebellum), and adipose tissue (mesenteric and interscapular brown fat). In some rats (13 AL and 11 CR), the retroperitoneal and epididymal adipose tissue were also removed. After dissection, each tissue was rapidly freeze-dried with alumina. Tongs precooled to the temperature of liquid N2 were stored at ~80°C. Weights were determined for the following tissues: soleus, plantaris, epimysitic, heart ventricles, spleen, kidney, lung, interscapular brown fat pad, retroperitoneal fat pad, and epididymal fat pad. After tissue dissection, the bladder was emptied using a syringe, and the urine volume was determined. The collected urine was stored at ~80°C until an aliquot was used to determine 2-[3H]DG content by liquid scintillation counting as described in Determination of plasma and urine 2-[3H]DG and tissue 2-[3H]DG-6-P.

Determination of plasma glucose concentration. Plasma (60 µl) was diluted with water (540 µl), deproteinized by adding 300 µl each of 0.3 N NaOH2, and 0.3 N ZnSO4, and centrifuged at 10,000 g (at 4°C for 10 min). Glucose concentration was determined in the supernatant by the glucose oxidase-peroxidase method (Sigma Procedure 510; St. Louis, MO).

Determination of plasma and urine 2-[3H]DG and tissue 2-[3H]DG-6-P. The 3H content in deproteinated plasma (200 µl) was determined by scintillation counting. Total counts were corrected for background and quenching interference.

Portions of tissues were weighed and homogenized in ice-cold 0.5 M perchloric acid using glass-on-glass homogenizers. Homogenates were centrifuged (10 min at 10,000 g and 4°C), and resultant supernatants were neutralized (pH ~7.4) with 5 M KOH plus 0.5 M triethanolamine hydrochloride and centrifuged. A portion of the neutralized extract was used to determine total radioactivity (2-[3H]DG + 2-[3H]DG-6-P). Another aliquot was mixed with equal volumes of 0.3 N Ba(OH)2, and 0.3 N ZnSO4. This mixture was centrifuged, and the supernatant was used to determine unphosphorylated
2-[^3]H DG levels (27). Quench corrections were made as for plasma. Tissue 2-[^3]H DG-6-P was calculated as the difference between total and 2-[^3]H DG values (12).

An aliquot of urine aspirated from the bladder was used to determine 2-[^3]H DG concentration [disintegrations·min⁻¹ (dpm)·ml⁻¹], and this value was multiplied by the urine volume to determine the urine 2-[^3]H DG content. This value was divided by the estimated amount (dpm) of 2-[^3]H DG injected into the animal to estimate the percent injected 2-[^3]H DG that was in urine.

Calculation of $R_9^\prime$. Glucose utilization by individual tissues was assessed by a modification (28) of the 2-DG method originally described by Sokoloff et al. (43). Plasma 2-[^3]H DG levels were fitted to a double-exponential equation for determination of the integral (Rstr; MicroMath Scientific Software, Salt Lake City, UT), as previously discussed (28). The values are referred to as $R_9^\prime$ (µmol·100 g tissue⁻¹·min⁻¹) and are calculated using the following equation (28)

$$R_9^\prime = \frac{C_p C_t}{\int_0^T C_p(t) dt}$$

where $C_p$ is mean plasma glucose concentration (µmol/ml) over the 45-min experimental period; $C_t(45)$ is tissue 2-[^3]H DG-6-P per unit tissue mass (dpm/100 g) at 45 min after tracer injection; and $\int_0^T C_p(t) dt$ is the area under the curve (AUC) for plasma 2-[^3]H DG over the 45-min period after tracer injection (dpm·ml⁻¹·min⁻¹). A detailed description of these calculations and the underlying assumptions has been published previously (28).

Determination of plasma insulin and C-peptide. Plasma insulin and C-peptide concentrations were measured by radioimmunoassays using rat insulin and C-peptide, respectively, as the standards (Linco, St. Louis, MO). Plasma from the 45-min sample was used for all but three rats (2 AL and 1 CR; the 0-min sample was used for these animals).

Determination of plasma FFA concentration. Plasma FFA levels were measured by a colorimetric assay (NEFA-C; Wako, Dallas, TX). Plasma sampled at the final time point was used for all but three rats (2 AL and 1 CR; the 0-min sample was used for these animals).

Determination of plasma lactate concentration. Plasma lactate, from the 0-min sample, was determined using a Yellow Springs LactateAnalyzer (model 1500 Sport).

Determination of tissue G-6-P and F-6-P concentrations. Liver and gastrocnemius G-6-P and F-6-P levels were measured by the fluorometric procedure previously described (29).

Determination of tissue glycogen concentrations. Gastrocnemius and soleus glycogen concentrations were determined by the amyloglucosidase method (36). Portions of the liver were hydrolyzed in 2 M HCl at 100°C for 2 h. Hydrolyzates were neutralized with 0.67 M NaOH, and glycogen was determined as previously described (36).

Statistical analysis. Differences between AL and CR tissue weights were analyzed using a one-tailed t-test. Diet-related differences for body weight and food intake were analyzed using one-way ANOVA for repeated measures (SAS, Cary, NC). The remaining data were analyzed by two-way ANOVA using the general linear models procedure; this analysis revealed the independent main effects of diet and time as well as their interaction. When a significant ($P < 0.05$) effect was found by ANOVA, Duncan’s multiple range test was performed to locate the source of significant variance.

**RESULTS**

Food intake, body weight, and tissue weights. Patent catheters were achieved for 75% of rats studied, and the presented data (means ± SE) are from these 30 animals (7 AL at 1500, 8 AL at 2100; 8 CR at 1500, 7 CR at 2100). Urokinase treatment established patency in four rats (1 AL at 1500, 1 CR at 1500, 2 CR at 2100), and their results did not differ from the rats in the same group that did not receive urokinase treatment.

As expected, the CR rats weighed ~60% as much as the AL rats throughout the experiment, and body weight was slightly less than baseline values after the surgery (4% in AL and 1.5% in CR rats when they were killed). Body weights were stable for at least 9 days before 2-DG injection and tissue collection. Final body weights were as follows: AL, 468.5 ± 9.1 and CR, 283.7 ± 19.9 g, $P < 0.0001$. On the day after surgery, AL rats consumed 47% of baseline intake; consumption increased to 86% of baseline by 3–4 days postsurgery and was 95% of baseline during the final 7 days of the experiment. Food intake of CR rats was not altered after surgery. During the baseline period, food intake by CR rats (11.3 ± 0.1 g/day) was 59% of the baseline values for AL rats (18.3 ± 0.5 g/day). During the 7 days before 2-DG injection, food intake of CR rats was 62% of AL rats during the same time period. The injection of 2-[^3]H DG was performed 12–19 days postsurgery, and this duration did not differ between AL and CR rats (12.9 ± 0.3 and 13.3 ± 0.6 for AL and CR, respectively).

Based on body weight and food intake values, the animals were in a steady state for at least 7 days before the final experiment.

The absolute weights (mg) of all tissues weighed were significantly lower in CR compared with AL rats (Table 1). There was a disproportionate difference in the weight of white adipose tissue compared with lean tissue. The percent difference for body weight in CR vs. AL rats was 39%, which was somewhat more than the percent difference for each of the lean tissues weighed (soleus = 28%, plantaris = 27%, epitrochlearis = 32%, heart = 26%, cerebellum = 17%, spleen = 35%; kidney = 29%, and lung = 24%) and considerably less than the

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weight, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>CR</td>
</tr>
<tr>
<td>Epitrochlearis 122.3 ± 4.7</td>
<td>83.3 ± 2.5*</td>
</tr>
<tr>
<td>Soleus       187.1 ± 6.8</td>
<td>135.0 ± 1.6*</td>
</tr>
<tr>
<td>Plantaris    405.3 ± 13.7</td>
<td>297.5 ± 7.5*</td>
</tr>
<tr>
<td>Heart (both ventricles) 1,059.2 ± 26.0</td>
<td>783.3 ± 28.8*</td>
</tr>
<tr>
<td>Spleen       940.5 ± 28.9</td>
<td>610.1 ± 25.6*</td>
</tr>
<tr>
<td>Kidney       1,297.4 ± 18.3</td>
<td>922.8 ± 13.0*</td>
</tr>
<tr>
<td>Lungs (both)  1,545.1 ± 63.8</td>
<td>1,176.7 ± 83.2*</td>
</tr>
<tr>
<td>Cerebellum   398.6 ± 22.7</td>
<td>331.5 ± 19.9*</td>
</tr>
<tr>
<td>Interscapular brown fat pad 274.1 ± 55.2</td>
<td>155.6 ± 21.8*</td>
</tr>
<tr>
<td>Retropitoneal fat pad 1,325.2 ± 147.0</td>
<td>189.0 ± 23.8*</td>
</tr>
<tr>
<td>Epididymal fat pad 1,634.3 ± 131.0</td>
<td>575.7 ± 60.7*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 11–15 rats/group. AL, ad libitum; CR, calorie restricted. Whole tissue weights for gastrocnemius, diaphragm, liver, and mesenteric fat were not measured. *Significantly different from AL value ($P < 0.05$).
percent difference in the white adipose depots measured (retroperitoneal fat = 86% and epididymal fat pad = 65%; the mesenteric fat pad is very diffuse, so we did not attempt to measure total mass). In contrast to white adipose depots, the 43% smaller mass of the brown adipose depot in CR rats was similar to the relative difference in body weight.

Plasma glucose concentration. Plasma glucose levels within each group were in a steady state during the 45-min sampling period (coefficients of variation <7%). The mean value for plasma glucose was determined from the samples taken 1, 3, 5, 10, 15, 25, and 45 min after 2-DG injection, and significant main effects of diet (AL greater than CR, P < 0.05) and time (2100 greater than 1500, P < 0.05) were found (Table 2). The AL 2100 group was significantly (P < 0.05) higher than all other groups (7% above CR 2100 values). The values for the CR 1500 group tended to be lower than the AL 1500 group (4%; not statistically significant).

Plasma insulin and C-peptide concentrations. Significant main effects of diet (AL greater than CR, P < 0.0001) and time (2100 greater than 1500, P < 0.05) on plasma insulin were found (Table 2). The CR groups were 75 and 58% lower than time-matched AL groups at 1500 and 2100, respectively. Insulin concentrations were higher at 2100 vs. 1500 for each diet group (28% for AL and 110% for CR). Similar results were found for C-peptide as follows: the CR groups were 68 and 53% lower than time-matched AL groups at 1500 and 2100, respectively. C-peptide levels at 2100 were higher than time-matched AL groups (7% above CR 2100 values). The values for the AL 1500 group tended to be lower than the AL 1500 group (4%; not statistically significant).

Plasma lactate concentration. There were no significant effects on plasma lactate, but there was a trend (P = 0.054) for a diet effect, with higher lactate values (24–38%) in the CR vs. AL groups (Table 2).

Plasma 2-DG disappearance. Figure 1 illustrates the disappearance of 2-DG from plasma and the calculated AUC for each group. Significant main effects of diet (AL greater than CR, P < 0.0001) and time (1500 greater than 2100, P < 0.05), and a significant interaction (P < 0.05), were found for the AUC. The AUC for each CR group was significantly lower than each AL group, and the CR 2100 group was also lower than the CR 1500 group.

Glucose utilization by individual tissues. Significant main effects of time (2100 greater than 1500) were evident for the R_g of gastrocnemius, soleus, and plantar muscles; the values at 2100 ranged from 32 to 113% higher than diet-matched values at 1500 (Table 3). There was a significant diet effect in the epitrochlearis (CR greater than AL, P < 0.05); the CR 2100 value was 88% higher than the value for each AL group. The plantar muscle for the CR 2100 group was 40% higher than the AL 2100 group, and there was a trend in the gastrocnemius for the CR 2100 values (which were significantly greater than each 1500 group) to exceed AL 2100 levels by 27%. There was no evidence for a CR-induced increase in the soleus. The R_g values for the diaphragm were unaffected by diet or time. The heart was unusual in that there was a significant interaction (P < 0.0005), with the AL 1500 values 31% greater than the CR 1500 values, whereas the converse was true at 2100 when the CR values were 27% higher than the AL values.

Significant main effects of diet (CR greater than AL) were evident for each adipose depot that was studied (Table 3). The relative magnitude of the increase for CR vs. AL was greatest in the retroperitoneal depot (~500%) and least in the epididymal depot (~100%); the CR-induced increases in the mesenteric and interscapular brown fat depots were intermediate in relative magnitude.

There was a significant main effect of diet on liver R_g (AL greater than CR, P < 0.05; Table 3). The CR values were 12–15% lower than time-matched AL values.

Among the organs studied that are not targets for insulin-stimulated glucose uptake (kidney, lung, spleen, and brain), there was no significant main effect of diet on R_g (Table 3). R_g rates for kidney and lung were not affected by time. There were significant main effects of time for spleen (2100 greater than 1500, P < 0.005) and cerebellum (1500 greater than 2100, P < 0.05). In the spleen, there was also a significant interaction (P < 0.05) attributable to the 13% decrease in CR 1500 vs.

Table 2. Plasma parameters from AL and CR rats at 1500 and 2100

<table>
<thead>
<tr>
<th></th>
<th>1500</th>
<th>2100</th>
<th>Main Effects</th>
<th>Interaction of Diet × Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AL</td>
<td>CR</td>
<td>AL</td>
<td>CR</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>7.37 ± 0.20*</td>
<td>7.06 ± 0.20*</td>
<td>7.97 ± 0.09*</td>
<td>7.40 ± 0.21*</td>
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<tr>
<td>Insulin, pM</td>
<td>208 ± 34*</td>
<td>53 ± 8†</td>
<td>267 ± 22*</td>
<td>111 ± 34†</td>
</tr>
<tr>
<td>C-peptide, pM</td>
<td>612 ± 74*</td>
<td>194 ± 25†</td>
<td>772 ± 82*</td>
<td>364 ± 89†</td>
</tr>
<tr>
<td>C-peptide-to-insulin ratio</td>
<td>3.1 ± 0.3</td>
<td>3.9 ± 0.4</td>
<td>2.9 ± 0.2</td>
<td>3.8 ± 1.0</td>
</tr>
<tr>
<td>Free fatty acids, mM</td>
<td>0.12 ± 0.02*</td>
<td>0.08 ± 0.02*</td>
<td>0.31 ± 0.08†</td>
<td>0.16 ± 0.05*</td>
</tr>
<tr>
<td>Lactate, mM</td>
<td>1.15 ± 0.07</td>
<td>1.58 ± 0.20</td>
<td>1.04 ± 0.06</td>
<td>1.29 ± 0.26</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–8 rats/group. Within a row, values without superscripts or values sharing a common superscript are not significantly different (P < 0.05) as determined by post hoc analysis. NS, not significant.
percentage of 2-DG was phosphorylated for CR vs. AL. Only the heart had a small but statistically significantly lower percentage of phosphorylated 2-DG in CR compared with AL rats. In the gastrocnemius, plantaris, and cerebellum, there was also a small but significant increase in percentage of phosphorylated 2-DG at 2100 compared with 1500.

**Table 3. Glucose metabolic index for tissues from AL and CR rats at 1500 and 2100**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>AL 1500 (P &lt; 0.05)</th>
<th>AL 2100 (P &lt; 0.01)</th>
<th>CR 1500 (P &lt; 0.05)</th>
<th>CR 2100 (P &lt; 0.005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>4.19 ± 0.33*</td>
<td>4.96 ± 0.33*</td>
<td>6.37 ± 1.00†</td>
<td>8.09 ± 1.14†</td>
</tr>
<tr>
<td>Soleus</td>
<td>27.42 ± 3.47</td>
<td>24.09 ± 3.27</td>
<td>36.17 ± 5.96</td>
<td>37.42 ± 4.29</td>
</tr>
<tr>
<td>Plantaris</td>
<td>6.13 ± 0.73*</td>
<td>6.28 ± 0.82*</td>
<td>9.59 ± 1.32*</td>
<td>13.40 ± 1.56†</td>
</tr>
<tr>
<td>Epitrochlearis</td>
<td>3.95 ± 0.83*</td>
<td>4.79 ± 0.39†</td>
<td>7.43 ± 1.61†</td>
<td>10.62 ± 2.47†</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>17.01 ± 1.37</td>
<td>18.44 ± 0.59</td>
<td>20.18 ± 1.61</td>
<td>21.45 ± 2.69</td>
</tr>
<tr>
<td>Heart</td>
<td>116.71 ± 8.24*</td>
<td>88.90 ± 2.60†</td>
<td>108.19 ± 8.41†</td>
<td>137.92 ± 7.43‡</td>
</tr>
<tr>
<td>Liver</td>
<td>1.15 ± 0.04</td>
<td>1.01 ± 0.07</td>
<td>1.15 ± 0.09</td>
<td>0.98 ± 0.04</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.77 ± 0.56</td>
<td>5.92 ± 0.44</td>
<td>5.91 ± 0.26</td>
<td>6.94 ± 0.43</td>
</tr>
<tr>
<td>Spleen</td>
<td>11.91 ± 0.48</td>
<td>10.40 ± 0.31†</td>
<td>12.64 ± 0.50*</td>
<td>13.16 ± 0.66*</td>
</tr>
<tr>
<td>Lung</td>
<td>6.12 ± 0.23</td>
<td>5.56 ± 0.36</td>
<td>6.16 ± 0.31</td>
<td>6.62 ± 0.27</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>20.17 ± 0.57</td>
<td>21.03 ± 0.50</td>
<td>19.24 ± 1.06</td>
<td>19.70 ± 0.65</td>
</tr>
<tr>
<td>Mesenteric fat</td>
<td>1.23 ± 0.27*</td>
<td>5.43 ± 0.61†</td>
<td>1.51 ± 0.34*</td>
<td>8.07 ± 2.79†</td>
</tr>
<tr>
<td>Interscapular brown fat</td>
<td>9.89 ± 4.99*</td>
<td>38.69 ± 12.94†</td>
<td>4.57 ± 1.73*</td>
<td>12.31 ± 2.81*</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>1.00 ± 0.27†</td>
<td>1.76 ± 0.47*</td>
<td>0.64 ± 0.05†</td>
<td>1.55 ± 0.34*</td>
</tr>
<tr>
<td>Retroperitoneal fat</td>
<td>0.92 ± 0.15*</td>
<td>5.01 ± 0.73†</td>
<td>0.87 ± 0.07*</td>
<td>5.76 ± 2.10†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–8 rats/group for all tissues except epididymal and retroperitoneal fat, where n = 5–8 rats/group. Units are μmol·100 g⁻¹·min⁻¹. Within a row, values without superscripts or values sharing a common superscript are not significantly different (P < 0.05) as determined by post hoc analysis. NS, not significant.
**DISCUSSION**

Moderate CR enhances insulin’s ability to increase glucose uptake in intact rats, in isolated adipocytes, and in isolated skeletal muscle preparations (4, 5, 23, 39). In those earlier experiments, glucose and/or insulin were adjusted so that AL and CR animals did not differ for either parameter. The major goal of the present study was to evaluate the influence of a prolonged period of moderate CR on glucose metabolism by individual tissues using unanesthetized rats without infusing exogenous glucose and/or insulin. In other words, we were interested in studying the effects of CR on glucose metabolism under usual living conditions. In this context, it was important that, after catheter placement, food intake was only transiently reduced, both food intake and body weight were in a steady state for at least 1 wk before the metabolic measurements, and movement by the rats about the cage was not restrained.

The disappearance rate of plasma 2-DG was faster in CR compared with AL rats, as indicated by lower AUC values for CR vs. AL rats. The lack of differences among groups in the loss of 2-DG in urine indicates that the diet-related differences were caused by tissue uptake. At 1500, $R_g$ (per unit tissue) was increased in CR vs. AL rats only in adipose tissue. In light of the small amount of adipose tissue in CR rats, other tissues must account for most of the CR-induced increase in plasma disappearance of 2-DG. The shift in body composition favored a greater rate of 2-DG disappearance in CR compared with AL animals because white adipose tissue has a relatively low rate of glucose uptake. Although it seems unlikely, it also remains possible that CR caused an increased 2-DG uptake by tissues other than those studied. The greater 2-DG clearance in CR vs. AL rats at 2100 corresponds with higher $R_g$ values for cardiac and skeletal muscle in CR compared with AL animals at this time. Our results supported...
part of our hypothesis: plasma 2-DG clearance at 2100 was faster in CR compared with AL rats, concomitant with higher $R_g$ values in several skeletal muscles from the CR vs. AL animals. However, in contrast to our expectations (that 2-DG clearance and muscle $R_g$ would be greater in AL vs. CR rats at 1500), 2-DG clearance was faster in CR compared with AL rats at 1500, and skeletal muscle $R_g$ was similar in CR and AL rats at this time.

Glucose transport is the rate-limiting step for glucose metabolism by skeletal muscle under most conditions, so this process is probably important for the CR-induced increase in 2-DG clearance. Basal glucose transport (without insulin) in isolated skeletal muscle is unaffected by CR (3, 4), and a CR-induced increase in insulin-stimulated glucose uptake is well documented in isolated or perfused muscles (3, 4, 6, 8, 23). The significantly higher $R_g$ in plantaris and epitrochlearis muscles of CR vs. AL rats and the trend for a CR-induced increase in gastrocnemius $R_g$ at 2100 are consistent with enhanced insulin action with CR. The lack of a decrease in $R_g$ in soleus and diaphragm, despite 58–75% lower insulin in CR vs. AL animals, might also have been caused, at least in part, by greater effectiveness of insulin in CR rats. Indeed, enhanced insulin-stimulated 2-DG uptake has been reported in isolated diaphragms from CR rats (6), and we have found that CR leads to enhanced insulin-stimulated 2-DG uptake in isolated soleus muscles from male mice (Gazdag and Cartee, unpublished observation).

In addition to insulin, contractile activity is an important stimulus for skeletal muscle glucose transport. In all of the hindlimb muscles we studied, $R_g$ values were somewhat higher at 2100 vs. 1500, coinciding with the greater physical activity of rats during the dark portion of the day (11) and with the time-related increase in $R_g$ values for muscles from AL rats (19). Others have reported that daily locomotor activity is slightly higher in CR compared with AL rats (11, 45), with peak activity for CR rats occurring when they are

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**Fig. 2.** Mean glucose 6-phosphate concentrations in gastrocnemius (A) and liver (B) of AL and CR animals at each time point. Values are means ± SE ($n = 7$–$8$/group). For gastrocnemius, there was a significant main effect of diet ($P < 0.01$) and time ($P < 0.0005$); for liver, there was a significant main effect of time ($P < 0.01$). Significant differences ($P < 0.05$) between groups as detected by post hoc analysis are denoted by different letters (groups sharing a letter are not different).

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**Fig. 3.** Mean fructose 6-phosphate concentrations in gastrocnemius (A) and liver (B) of AL and CR animals at each time point. Values are means ± SE ($n = 7$–$8$/group).
comprised predominantly of fast-twitch fibers (epitroch-

eases that are typical for 12-mo-old rats. The higher $R_8$ values among the muscles studied, it is notable that at neither time, in any skeletal muscle studied, was $R_8$ lower for CR vs. AL rats.

It is striking that $R_8$ was increased by CR only in tissues that are known to express the GLUT-4 glucose transporter protein (white adipose tissue and BAT and skeletal and cardiac muscle; see Ref. 37). In skeletal muscle, CR does not increase the total abundance of GLUT-4 (4), but we recently demonstrated that CR enhances GLUT-4 function by increasing the amount of GLUT-4 translocated to the cell surface in response to insulin (7).

$R_8$ has been used to study tissue glucose metabolism under many physiological conditions, including exercise (24), weaning (21), diurnal changes (18), and fasting (22). The methods, assumptions, and rationale for measuring $R_8$ have been described in detail (28). In accordance with the assumptions of the method, arterial glucose values were in a steady state during the 2-DG experiment. As previously discussed by Kraegen and colleagues (28), $R_8$ is an index, rather than an absolute measurement, of glucose uptake. In their classic study, Sokoloff et al. (43) demonstrated that, in the rat brain, the rate of 2-DG uptake is ~50% of glucose uptake. Ferré et al. (12) reported that 2-DG uptake rate in skeletal muscle was ~100% of the glucose uptake rate, and 2-DG uptake by white adipose tissue was ~60% of glucose uptake; in both tissues, insulin caused 2-DG and glucose uptake to increase to a similar extent (12). Previous studies have shown that CR results in comparable relative increases in skeletal muscle uptake of glucose (23), 2-DG (6), and 3-O-methylglucose (4). An assumption when using $R_8$ is that 2-DG-6-P is trapped in the cell. Even if CR causes a decrease in glucose 6-phosphatase activity, it would likely have little effect on 2-DG-6-P accumulation because, even in AL rats, the activity of this enzyme is very low in skeletal muscle. At least in skeletal muscle, it seems reasonable to use $R_8$ as an index of glucose metabolism in CR rats.

Skeletal muscle is likely responsible, in large part, for the increased respiratory quotient (RQ) found in CR vs. AL rats during the first several hours after CR rats are fed (35). By contrast, during the light portion of the day, RQ values are lower in CR than AL rats (35), indicating proportionally lower carbohydrate oxidation in CR animals. At 1500, only myocardial and splenic $R_8$ was somewhat lower for CR vs. AL rats, and glucose uptake by these tissues cannot account for the previously reported difference in RQ. The lack of diet-related differences for $R_8$ in skeletal muscles at 1500, together with the previously reported lower RQ in CR compared with AL rats, suggests that a smaller fraction of the glucose cleared from the blood was oxidized in CR vs. AL rats at 1500. It is interesting that at this time, plasma lactate, a product of nonoxidative glucose metabolism, tended to be 38% higher in CR vs. AL rats.

fed (11). We did not systematically measure physical activity, but when CR rats were fed (at 1800), they appeared to be more active than AL animals. During and after 2-DG injection (at 1500 and 2100), however, we did not detect any obvious differences in physical activity between the diet groups; at those times, animals were engaged in the types of nonvigor activities that are typical for 12-mo-old rats. The higher $R_8$ with CR occurred only in skeletal muscles that are composed predominantly of fast-twitch fibers (epitroch-

![Fig. 4. Mean glycogen concentrations in gastrocnemius (A), soleus (B), and liver (C) of AL and CR animals at each time point. Values are means ± SE (n = 7–8/group). For gastrocnemius, there were a significant main effect of diet (P < 0.01) and a significant interaction (diet × time; P < 0.005); for soleus, there were significant main effects of diet (P < 0.0001) and time (P < 0.005) and a significant interaction (diet × time; P < 0.05); for liver, there were significant main effects of diet (P < 0.0005) and time (P < 0.0001). Significant differences (P < 0.05) between groups detected by post hoc analysis are denoted by different letters (groups sharing a letter are not different).](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00380.2017)
Several other findings in the current study provide further indirect evidence that CR might have altered posttransport steps in glucose metabolism in some tissues. Gastrocnemius G-6-P concentration was significantly reduced in CR compared with AL animals despite a trend for increased R_g at 2100 and no change in the percentage of 2-DG in the phosphorylated form, suggesting an enhanced rate of G-6-P removal. Glucose incorporation into glycogen is an important route of glucose disposal, and gastrocnemius glycogen concentration was higher in CR compared with AL at 2100, implicating an effect on glycogen synthase. Earlier research demonstrated that total glycogen synthase activity in skeletal muscle was unaltered by reduced calorie intake (41), so our working hypothesis is that CR enhances the insulin-induced activation of this enzyme. For the epitrochlearis and BAT, the proportion of 2-DG in the phosphorylated form (2-DG-6-P) was ~10–20% greater for CR vs. AL values, which is consistent with a CR-related increase in hexokinase activity in these tissues.

Core temperature is ~2°C lower for CR vs. AL rats (11). A low core temperature has been suggested to selectively activate the sympathetic nerves innervating BAT (17), and sympathetic activity is a potent stimulus for glucose uptake by BAT (44). Therefore, the higher R_g values in BAT of CR rats might be the result of an increase in sympathetic activation of BAT. The relative magnitude of the CR-related difference in glycemia at 2100 (7%) corresponds with the ~4–10% CR-induced decrease found by Masoro et al. (33) in unanesthetized rats at approximately the same time. At 1500, we found a 4% lower glucose concentration in CR vs. AL rats, compared with the ~10–20% difference at a similar time reported by Masoro and colleagues. We measured arterial glucose concentration, while their samples were obtained by cutting the tip of the tail. We have also found an ~10–20% CR-induced decrease in glucose concentration of blood collected by cutting the tip of the tail of anesthetized rats (3, 7, 8). With that procedure, both arterial and venous blood are collected. Venous glucose concentration is influenced by tissue rates of glucose metabolism and blood flow. It seems unlikely that glucose metabolic rate by the tail is greatly altered by CR; however, blood flow to the tail, a major site for heat dissipation in rats (38), can vary markedly. Therefore, if blood flow to the tail is lower in CR rats, perhaps to minimize heat loss in light of their reduced core temperature (11), the magnitude of the diet-related difference in glucose concentration for mixed arterial-venous samples from the tail would be greater than the diet-related difference in arterial samples. In any case, the availability of a substantial amount of hepatic glycogen in CR rats at 1500 would be expected to prevent profound hypoglycemia, consistent with our results.

Maximal life span of rats, as well as other species, is increased by CR (42). The amount of life extension is related to the degree of energy restriction, provided malnutrition is avoided, and not to the reduced intake of any individual nutrient. CR also delays or prevents a number of age-related pathologies (31). The mechanisms for the antiaging actions of CR are unknown, but much interest has focused on the role of altered energy metabolism. It has been suggested that a reduction in metabolic rate causes the CR-induced prolongation of life (40). Indeed, reducing calorie intake by ~40% for ~6 wk has been shown to reduce metabolic rate (per kg lean body mass or per kg^0.75) of rats (15, 34). However, energy expenditure (per kg lean body mass or per kg^0.75) and mean 24-h RQ have been reported to be similar for AL and CR (40% reduction in calorie intake) rats when the energy restriction was continued for ~4 mo (11, 31, 35). Our data provide novel insights into the metabolic effects of CR at the level of individual tissues. There was no evidence that 8 mo of CR altered the glucose uptake by the kidney, lung, or cerebellum (i.e., tissues not sensitive to insulin-stimulated glucose uptake), and R_g values for skeletal muscles and adipose tissues were as high or even higher for CR compared with AL rats. These observations are consistent with the concept that CR does not reduce energy flux and argue against the idea that the effects of 8 mo of CR on these tissues are attributable to lower glucose uptake.

It has been hypothesized that some of the beneficial effects of CR are mediated via lesser glycation of proteins and DNA, as a consequence of reduced glycemia. Of course DNA is intracellular, as are most proteins, and intracellular hexose concentration would not be expected to decline if glucose entry is not reduced by CR, unless glucose metabolism is altered at postuptake steps. In this context, the CR-induced reduction in G-6-P concentration of skeletal muscle is notable because, unlike glucose (which is found at very low intracellular concentrations in muscle), G-6-P is found at relatively high intracellular concentrations. Furthermore, at equimolar concentrations, G-6-P is three times as effective as glucose in generating advanced glycation end product fluorescence (16), presumably because, in a physiological solution, glucose is almost entirely in the stable ring configuration (2), whereas a greater portion of G-6-P exists in the more reactive, open-chain form (30). It remains to be determined if the lower G-6-P is important for other CR-induced adaptations in skeletal muscle. The effect of CR on G-6-P in skeletal muscle should not be extrapolated to other tissues, e.g., hepatic G-6-P concentration was not reduced by CR.

A significant diet times time interaction for R_g was found only in heart and spleen; the differences among groups were quite small for the spleen. In the heart, the 27% lower R_g value in CR vs. AL at 1500 was reversed at 2100, when the R_g was 31% greater for CR vs. AL rats. Perhaps the doubling of insulinemia in CR rats at 2100 vs. 1500 accounts for their increase in myocardial R_g at 2100. Hapatic R_g values for CR rats were 12–15% lower than time-matched AL values. However, because of the high glucose 6-phosphatase activity in the liver (consistent with the low percent of phosphorylated 2-DG in this tissue), hepatic R_g values cannot be used as an index of glucose uptake rates (20, 25). The kidney also has relatively high glucose 6-phosphatase activity, consistent with the relatively low portion of 2-DG-6-P.
Our values for percent phosphorylated 2-DG in other tissues are comparable to previously published results, e.g., the percent phosphorylated 2-DG in skeletal muscles (62–87%) corresponds with the range (53–81%) reported by Hom et al. (20).

Plasma FFA concentration tended to be ~20–50% lower in the CR vs. the AL group, but there was not a significant diet effect. We previously found no effect of brief (5 or 20 days) CR on circulating FFA in 24-mo-old female rats (8), and FFA concentration was higher than AL values in 19-mo-old male rats after 20 days but not ~15 mo of CR (9). The effect of CR on FFA levels might depend on gender and the severity, duration, and age of onset of CR.

Plasma insulin and C-peptide concentrations were lower in CR compared with AL rats, the C-peptide-to-insulin ratio tended to be higher in CR animals, and C-peptide and insulin values were highly correlated. Feuers and colleagues (13) observed greater insulin C-peptide and insulin values were highly correlated. Apparently reduced insulin secretion is the major cause for the lower insulin concentration with CR, and increased hepatic clearance might play a secondary role.

In conclusion, we found that the rate of plasma 2-DG disappearance was greater in CR compared with AL rats, despite much lower plasma insulin values in the CR animals. Our results indicate that 1) in many tissues, CR had no apparent effect on vivo 2-DG uptake; 2) no tissue did CR reduce 2-DG uptake at both times studied; 3) CR-related increases in 2-DG uptake occurred only in tissues that are known to express GLUT-4 (white adipose tissue, BAT, and cardiac and skeletal muscle); and 4) CR can apparently, in addition to enhancing glucose transport, alter posttransport aspects of in vivo glucose metabolism in skeletal muscle. On the basis of these results, it seems unlikely, at least in a number of tissues, that a reduced rate of glucose uptake is essential for the diet-related adaptations that occur in rats with 8 mo of moderate CR.

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