Effects of Changes in Calorie Intake on Intestinal Nutrient Uptake and Transporter mRNA Levels in Aged Mice

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In aged, chronically calorie-restricted (CR) mice, intestinal nutrient uptake is significantly higher than in same-age ad libitum controls. Can this chronic restriction-induced enhancement of uptake be reversed by ad libitum feeding? We addressed this question by switching 32-mo-old chronically CR mice to ad libitum feeding for 4 wk (CRAL).

Intestinal transport rate and total intestinal absorptive capacity for D-sugars and several nonessential L-amino acids decreased significantly in CRAL mice. In contrast, switching CR mice to an ad libitum regimen for only 3 d had no effect on intestinal nutrient transport, indicating that the negative effects of ad libitum feeding require a duration longer than the 3-d lifetime of most enterocytes. Permeability of the intestinal mucosa to L-glucose was independent of the switches in diet. Levels of the brushborder glucose transporter SGLT1, brushborder fructose transporter GLUT5, and basolateral sugar transporter GLUT2 mRNA as determined by reverse transcriptase-polymerase chain reaction in 6-, 24-, and 32-mo-old mice were each apparently independent of caloric restriction and age. We conclude that the high rates of intestinal nutrient uptake exhibited by chronically CR mice can be reversed by ad libitum feeding of only 1 mo duration. These decreases in uptake were due mainly to specific decreases in transport per unit weight of intestine and not to nonspecific decreases in intestinal mass. Changes in rates of sugar uptake induced by chronic CR and age are apparently not accompanied by changes in steady-state levels of mRNA coding for those transporters.

STUDIES on calorie restriction (CR) are highly relevant to aging because CR is the only method known to retard aging processes in mammals and to extend both mean and maximum life span in rodents (Masoro et al., 1991). Restriction of macronutrient levels (protein, fat, or carbohydrate) is a much less effective modulator of life span (Turturro et al., 1993). A 24-mo-old (median life span) mouse calorie restricted to 60% of ad libitum since 4 mo of age would have consumed over a span of 20 mo approximately 1.3 kg less food than a same age mouse fed ad libitum (AL); 1.3 kg is 65 times the body weight of a CR C57BL6 mouse. Such a large disparity in amounts of food processed affects intestinal enzyme and transporter levels (Ferraris and Diamond, 1997). Because the small intestine is the only site for nutrient digestion and absorption, studies of gut function during CR may increase our understanding of adaptive mechanisms underlying the age-retarding effects of CR.

Only recently did the small intestine receive attention in studies on CR. We have previously shown that, in aged (24 mo old) mice that had been calorie restricted since 4 mo of age, intestinal transport of D-sugars and several amino acids was higher than that in same age AL controls and was comparable to that of young mice fed AL (Ferraris et al., 1993; Casirola et al., 1996). CR of several months duration is probably required before increases in intestinal nutrient transport can be demonstrated because CR of 1 mo duration has little effect on intestinal nutrient transport in aged mice. Preliminary and ongoing studies involving young mice recently introduced to CR also suggest that prolonged (≥24 d) CR is necessary to demonstrate a significant change in intestinal nutrient absorption (Cao and Ferraris, unpublished observations). We now address the question whether higher rates of intestinal nutrient uptake observed in aged mice undergoing chronic CR are affected by AL feeding. If this remarkable adaptation to chronic CR takes a very long time to develop, can it be retained even if dietary regimen changes, or will this adaptation be reversed? If it is reversible, what is the time course, and does the time course parallel that of the change in body weight elicited by the switch to AL feeding?

Similar questions have been asked before with regards to the effect of age and CR on the specific activity of intestinal enzymes. Holt et al. (1991) showed that specific activities of sucrose, maltase, and alkaline phosphatase were each reduced during senescence, but CR delayed this age-related fall in enzyme activity. Age-related decreases in levels of enzyme and transporter proteins parallel these decreases in enzyme and transporter activities (Holt et al., 1991; Ferraris et al., 1993). Although there is meager information on the effects of age and CR on intestinal function, there is virtually no information on their effects on levels of enzyme and transporter mRNA; hence, this initial attempt to survey age-related and CR-related changes in mRNA levels of intestinal sugar transporters.

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In this study, we analyzed the effect of a switch to AL caloric intake on uptake of different nutrients. We used 3 d and 4 wk as two intervals after switching to AL caloric
intake. In a parallel study, we also determined the effects of age and CR on steady-state levels of the brushborder glucose (SGLT1), the brushborder fructose (GLUT5), and the basolateral glucose and fructose (GLUT2) transporter mRNA in the small intestine.

Materials and Methods

Animals and Diets
Male virgin C57BL mice, 32 mo of age, were obtained from the National Institute on Aging (NIA) breeding colonies. They began on a stepwise food intake reduction at 14 wk of age, and completed to 60% of their AL caloric intake by the 16th week and continued thereafter at that level. In our facility, they received 2.5 g/d of a complete, sterile diet [NIA P-31 for CR mice (for diet composition, see Casirola et al., 1996)]. We wanted to use an age group that has undergone CR for the longest time, and 32 mo was chosen because these mice have been calorie restricted for the 28 mo at the time of the experiment. No other older age group was available. Mice were housed in microisolators at 25 °C, on a 12/12 h light/dark schedule, and their body weights were stabilized for at least 1 wk before the switch in diets. Four weeks before the experiment, half of several batches of CR mice were switched to AL diet (regular NIA P-31; composition given in Casirola et al., 1996) (CRAL), and the other half remained calorie restricted (CR1). In a second series of experiments, half of several batches of CR mice were acutely switched to AL feeding for 3 d and were killed on the fourth day (ACRAL); the other half remained calorie restricted (CR2). We tested a 4-wk period to study long-term changes and a 3-d period to study short-term changes based on the average life span of enterocytes, which is less than 3 d in 6- and in 24-mo-old healthy, AL-fed mice of the same strain (Ferraris and Vinnakota, 1995).

Other studies have shown that enterocyte life span in mouse and rat small intestine is brief and is on the order of about 2 d (Ferraris et al., 1992; Atillasoy and Holt, 1993); CR for 1 mo reduces these rates of cell proliferation, but only by 25% (Lok et al., 1988).

Food consumption and body weights were monitored twice a week. Experiments were always carried out between 1100 and 1500 h to minimize circadian variations. Mice were not fasted before the experiment. They were anesthetized and subsequently killed by intraperitoneal injection of sodium pentobarbital (3.5 ml/kg body weight). The small intestine was gently flushed with cold saline, excised, and everted on a glass rod. Except for small portions of the proximal and distal small intestine that were saved for different assays, proximal (up to 12 cm distal to the pylorus), distal (up to 12 cm proximal to the caecum), and middle (about 50% of total intestinal length) small intestine were used for the preparation of sleeves.

In the third series of experiments, C57BL mice aged 6, 24, and 30 mo (or over 30 mo) were used in mRNA studies to represent young, aged, and senescent mice, respectively. Each age had AL or chronically CR representatives. Mice were obtained from NIA colonies as before and maintained under the same conditions as other mice in the study. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

Nutrient Transport Measurements

Everted intestinal sleeves, 1 cm long, prepared by the method of Karasov and Diamond (1983), were mounted on a steel rod (4 mm diameter), preincubated, and then incubated over a magnet bar stirring at 1200 rpm (to minimize unstirred layers) in Ringer solution (128 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 2.2 mM KH2PO4, 1.2 mM MgSO4, and 20 mM NaHCO3, pH 7.3 – 7.4) bubbled with 95% O2/5% CO2 in Ringer buffer at 37 °C, added with each test nutrient. We measured the uptakes of two sugars, [14C]D-glucose and [14C]D-fructose, and various amino acids representing different classes of transporters in mice (Karasov et al., 1986; Casirola et al., 1996): [3H]L-proline, [3H]L-alanine, [3H]L-glutamine, [3H]L-aspartate, [3H]L-leucine, and [3H]L-lysine. For each nutrient, uptake was measured at the concentration yielding its Vmax (50 mM for D-glucose, D-fructose, L-proline, L-alanine, L-glutamine, L-leucine; 25 mM for L-lysine and L-aspartate), because changes in Vmax are least affected by unstirred layers (Karasov and Diamond, 1983). Concentrations that yield Vmax are independent of age (Ferraris et al., 1993) and of starvation (Diamond and Karasov, 1984). Incubation times were 1 min for D-glucose and 2 min for D-fructose and all the amino acids.

Sleeves used for D-glucose, D-fructose, and L-proline were from proximal, middle, and distal small intestinal regions as previously defined by Casirola et al. (1996). All other sleeves were from randomized positions of the middle small intestine. D-sugar transport was simultaneously corrected for adherent fluid and for the diffusive component by measuring the transport of [3H]L-glucose (Karasov and Diamond, 1983). [3H]Polyethylene glycol (molecular weight, 4000) was used to correct for [3H]amino acids in the adherent fluid. Hence, we measured mediated D-sugar transport and total (mediated plus diffusive) amino acid transport.

All radioisotopes were from DuPont/NEN (Boston, MA), except for [3H]leucine and [3H]proline, which were from Amersham (Arlington Heights, IL).

Enterocyte Collection and Total RNA Preparation

Briefly, the small intestine was isolated, initially flushed with ice-cold phosphate-buffered saline (PBS) (130 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4), flushed twice with ice-cold PBS plus 1 mM dithiothreitol, placed in ice-cold PBS, divided into the most proximal and distal 10 cm sections, and then everted. Enterocytes were collected from each small intestinal region according to Ferraris et al. (1992), then stored at –80 °C. For each experiment, six mice each were used in 6- and 24-mo-old CR and AL fed groups, and their enterocytes were pooled. Because of very limited availability, three mice that were ≥30 mo old and AL fed and four mice that were ≥30 mo old and calorie restricted were used for each experiment.

Total RNA was prepared from these enterocytes by the method of Chomczynski and Sacchi (1987). Briefly, entero-
cytes were denatured, mixed with 2 M sodium acetate (pH 4), followed by phenol and then by chloroform/isoamyl alcohol, placed on ice, and then centrifuged. Total RNA in the upper aqueous phase was precipitated with isopropanol, and the RNA pellet was rinsed with ethanol and subsequently dissolved in 100 μl diethyl-pyrocarbonate (DEPC)-treated water. About 500 μg total RNA were collected from 200 mg cells from each sample. Subsequent analysis shows no RNA degradation and absence of contaminating DNA. Ratio of A260 to A280 for all samples is between 1.7 and 1.9.

Reverse Transcriptase — Polymerase Chain Reaction (RT-PCR); Primer Design

Mouse SGLT1 primers were designed based on the mouse cDNA sequences provided by Eric Turk (UCLA, personal communication). Sense (5') primer 5'-TGCTGTACAATGGAATCTCG-3' and antisense (3') primer 5'-CTGTACAAATGGTGGTGAGGA-3' were designed and a 617-bp PCR product was expected.

Mouse GLUT2 primers (Asano et al., 1989) were designed as sense (5') primer 5'-GGGATGAGGCTGAGAAGG-3' and antisense (3') primer 5'-GAAGATCTAGATGGTGGTGAGGA-3'. A 487-bp product was expected.

Because the mouse cDNA sequence for GLUT5 has not been reported yet, primers were designed according to rat cDNA sequence for GLUT5. The 3' homologous amino acid sequence among three species (human, rat, and pig; Kayano et al., 1990; Rand et al., 1993) was chosen. This sequence is at the same time unique for GLUT5 when compared with the homologous sequences for other GLUT transporters (Baldwin, 1993). The sense (5') primer was 5'-AGGAGGATGAGGCTGAGAAGG-3' and the antisense (3') primer was 5'-GAAGATCTAGATGGTGGTGAGGA-3'. A 306-bp product was expected.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to demonstrate the absence of age or diet effects on a constitutively expressed “housekeeping” gene. We have previously shown in rats that GAPDH is independent of age and diet (Shu et al., 1997). Because GAPDH genes are highly conserved in different species (Tso et al., 1985), the 3' conserved rat cDNA region was used to design mouse GAPDH-specific primers. Sense (5') primer 5'-TAAGCTCACCTGGCATGGCCCTCAG-3' and antisense (3') primer 5'-CATGAGGTCCACCACCCTGTT-3' were designed and a 908-bp PCR product was expected.

First-strand cDNA synthesis was performed following the manufacturer's protocol (BRL; Gaithersburg, MD), with oligo(dT) 12-18 as a primer to yield full-length cDNA. The 20-μl reactions were carried out under optimal conditions using 200 units SuperScript II RT (BRL). Total RNA from enterocytes of either proximal and distal small intestine of 6-, 24-, and 230-mo-old AL or CR mice (total of 12 samples) were each carefully quantified, and 5 μg of each was used as a template for the first-strand cDNA synthesis. After inactivating the reverse transcriptase by heating the samples at 95 °C for 10 min, the 20-μl reaction was diluted to 50 μl with sterile distilled water.

PCR amplification of these samples was performed using the paired primers described above. Reaction mixtures contained 1 μl of each paired primers (200 ng/μl), 1× PCR buffer, sterile distilled water, 1 mM MgCl₂, 2 mM dNTPs, and 2 units of Taq polymerase (Perkin-Elmer). The 38 μl master mixture was aliquoted into each tube of a set of 12. Two microliters of diluted first-strand cDNA reaction from each mouse treatment group were added to the corresponding tube. All these independent amplifications were carried out in the same PCR thermal cycler under the same conditions. Amplification parameters were 94 °C for 30 s; 65 °C for 30 s, and 72 °C for 1 min, for a total of 40 cycles. After completion, the reaction tubes were frozen at −20 °C and the mineral oil overlay was removed. Ten microliters from each of the 12 test tubes were loaded onto a 1% agarose gel and electrophoresed at 80 V for 2 hr. A 1-kb DNA ladder was used as a marker. The gel was then stained with ethidium bromide and photographed using Polaroid film. PCR fragments were quantified by UV densitometry (IS-1000 Digital Imaging System).

Northern Blot Analysis

Poly(A)⁺ RNA was extracted from total RNA pooled from the above samples. Because only a single Northern blot analysis can be made from this pooled sample and to support the RT-PCR findings which lacked internal controls, a separate batch of mice (three 6-mo-old and three 24-mo-old mice) was used for additional paired experiments on the effect of age on transporter mRNA levels. Poly(A)⁺ RNA from each sample (10 μg) were subjected to 1% agarose/6% formaldehyde electrophoresis and then transferred to a nitrocellulose membrane by capillary action. 32P-labeling of cDNA probes (rat GLUT5, rat GLUT2, and rabbit SGLT1), hybridization of labeled probes to membranes, washing procedures, and exposure of x-ray films were described in detail previously (Shu et al., 1997).

Statistical Analysis

To minimize the effects of experimental variation on comparisons, one mouse from the CR1 group and one from the CRAL group, or one mouse from the CR2 group and one from the ACRAL group, were used in each uptake experiment. Results were expressed as means ± SE (n). Time-related changes in daily food consumption and the differences in body weight, intestinal weight, and length were each analyzed by one-way analysis of variance (ANOVA). The simultaneous effects of diet switch and intestinal position on uptake rate were analyzed by two-way ANOVA, and those of diet, intestinal position, and age on transporter mRNA levels were analyzed by three-way ANOVA. If diet effects were significant (p ≤ .05) and if the p-value for interaction was significant (p ≤ .05) or was approaching significance (.05 < p < .10), a one-way ANOVA was also used to determine the effect of diet switch on uptake per mg and per cm in sleeves from the same intestinal region. This type of follow-up analysis by one-way ANOVA is equivalent to a follow-up analysis by Student’s t-test. Finally, the effect of CR on total absorptive capacity for D-glucose, D-fructose, and L-proline was analyzed by one-way ANOVA. Statistical analysis was done using the Statgraphics Program (Statistical Graphics Corp., Princeton, NJ), whereas the power of our statistical com-
parisons was estimated using Statistical Power Analysis (Lawrence Erlbaum Associates, Mahwah, NJ).

The following are examples of the types of differences we can reliably detect with our sample sizes. For glucose uptakes per mg in the middle intestine, an $n = 8$ per diet allowed us, at minimum, to detect a difference between diets of 2.4 nmol/mg·min with 86% power. For fructose and proline uptakes per mg in the same regions, an $n = 8$ also allowed us to detect a difference of 0.42 (power = 78%) and 1.85 (power = 86%) nmol/mg·min, respectively. For glucose, fructose, and proline uptakes per cm in the middle intestine, an $n = 8$ allowed us to detect a difference of 120 (power = 85%), 12 (power = 86%), and 70 (power = 70%) nmol/cm·min, respectively.

**RESULTS**

**Effects of Switch in Caloric Intake on Clinical Parameters**

At the time of the experiment, the body weight of CRAL mice was 135% higher than that of CR1 mice, and that of ACRAL mice was 121% higher than that of CR2 mice; both differences were significant (Table 1). The time course of changes in body weight of CR1 and CRAL mice over a 4-wk period is shown in Figure 1A. The initial weight of CR2 and ACRAL mice before the switch was 20.7 ± 0.6 g, which is similar to the final weight of CR2 mice but is significantly less ($p < .001$) than that of ACRAL mice.

AL-fed mice aged 6, 24, and 30+ mo that were used for the RT-PCR experiments weighed 31.5 ± 2.9, 33.1 ± 0.7, and 36.7 ± 6.0 g, respectively. CR mice of the same age groups weighed 18.4 ± 1.7, 24.3 ± 1.1, and 24.4 ± 1.4 g, respectively. Thus, the body mass of 6-, 24-, and >30-mo-old CR mice were 41, 27, and 34%, respectively, less ($p < .001$) than AL mice of the same age.

Both CR1 (Figure 1B) and CR2 mice consumed their entire daily allowance of 2.5 g/d. After the switch, there was a significant difference in feeding rate between CRAL (3.79 ± 0.13 g/d) and CR1 mice (by one-way ANOVA, $p < .001$). Three days after the switch from CR to AL feeding, ACRAL mice had a feeding rate of 3.78 ± 0.32 g/d, which is also significantly greater ($p < .001$) than that of CR2 mice.

**Table 1. Effect of a Switch From Chronic CR to AL Feeding on Body Weight, Intestinal Weight, and Intestinal Length in 32-mo-old Mice**

<table>
<thead>
<tr>
<th></th>
<th>Final Body Weight (g)</th>
<th>Intestinal Weight (g)</th>
<th>Intestinal Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1</td>
<td>20.9 ± 1.1</td>
<td>1.6 ± 0.6</td>
<td>43.3 ± 1.3</td>
</tr>
<tr>
<td>CRAL</td>
<td>28.2 ± 4.8</td>
<td>1.9 ± 0.13</td>
<td>44.8 ± 1.6</td>
</tr>
<tr>
<td>p-value</td>
<td>.0001</td>
<td>.13</td>
<td>.51</td>
</tr>
<tr>
<td>CR2</td>
<td>20.2 ± .5</td>
<td>1.7 ± 0.05</td>
<td>42.9 ± .6</td>
</tr>
<tr>
<td>CRAL</td>
<td>24.5 ± .8</td>
<td>1.9 ± .11</td>
<td>43.4 ± .6</td>
</tr>
<tr>
<td>p-value</td>
<td>.0002</td>
<td>.08</td>
<td>.44</td>
</tr>
</tbody>
</table>

Notes: Values are means ± SE ($n = 8$). CR1 and CR2: calorie restricted since 3 mo of age; CRAL and ACRAL: calorie restricted since 3 mo of age; then switched to AL 4 wk or 3 d before experiment, respectively. Hence, CR1 and CRAL final weights were determined at 33 mo of age and that of CR2 and ACRAL at 32 mo of age.

Despite the dramatic differences in body weights, there was no difference in intestinal weight and length between CRAL and CR1 mice or between ACRAL and CR2 mice (Table 1). In mice used for RT-PCR experiments, intestinal weights of AL-fed 6-, 24-, and ≥30-mo-old mice were 1.35 ± 0.25, 1.77 ± 0.10, and 1.75 ± 0.09 g, respectively. For CR mice of the same age group, intestinal weights were 1.20 ± 0.05, 1.68 ± 0.22, and 1.63 ± 0.23 g, respectively. Hence, age ($p = .01$ by two-way ANOVA) but not caloric restriction ($p = .24$) had an effect on intestinal weight. In contrast, intestinal length was independent of age ($p = .09$ by two way ANOVA) and CR ($p = .27$).

**Transport of Sugars**

**D-glucose.** — By two-way ANOVA, D-glucose transport per mg (Figure 2A) was significantly influenced by the dietary switch from chronic CR to 1 mo AL feeding ($p = .0002$) and by intestinal region ($p < .0001$). The interaction between intestinal region and diet approached significance ($p = .095$). The mean glucose uptake per mg across intestinal regions was 3.31 ± 0.45 for CRAL mice and 5.95 ±
0.71 nmol/min for CR1 mice. D-Glucose uptake per mg in the CRAL mice was significantly lower than that of the CR1 in all intestinal positions (by one-way ANOVA, \( p = .02 \) in the proximal and middle small intestine and \( p = .01 \) in the distal).

D-Glucose transport per cm (Figure 2B) was also significantly affected by both diet switch and position (\( p = .006 \) and \( p < .0001 \), respectively). The interaction between region and diet also approached significance (\( p = .08 \)). The mean glucose uptake per cm across intestinal regions was 163 ± 26 for CRAL and 259 ± 38 nmol/min for CR1 mice. However, by subsequent one-way ANOVA, the effect of diet was significant only in the proximal small intestine (\( p = .05 \)) and not significant in the middle and distal small intestine (\( p = .09 \) and .11, respectively).

A switch to AL feeding of 3 d duration did not significantly influence D-glucose transport expressed per mg (\( p = .81 \) by two-way ANOVA) or per cm (\( p = .17 \)), whereas the effect of position was always highly significant (\( p < .0002 \), both as expressed per mg and per cm) (Table 2). Interactions between independent variables were not significantly different either per mg (\( p = .47 \)) or per cm (\( p = .24 \)).

**D-fructose.** — D-Fructose transport per mg (Figure 2C) was also highly significantly affected by the switch from chronic CR to AL feeding of 1 mo duration (\( p = .0006 \) by two-way ANOVA) and by intestinal position (\( p < .0001 \)). The interaction between region and diet approached significance (\( p = .08 \)). Fructose transport per mg across intestinal regions was 1.38 ± 0.15 and 0.86 ± 0.09 nmol/min for CR1 and CRAL mice, respectively. In the proximal small intestine, D-fructose uptake in CRAL mice was significantly lower than that in CR1 mice (\( p = .006 \) by one-way ANOVA); in the middle and distal small intestine, the uptake in CRAL mice tended to be lower than that of CR1 mice, but statistical significance was not achieved (\( p = .09 \) and \( p = .12 \), respectively).

When D-fructose transport was expressed per cm (Figure 2D), the effect of the switch in caloric intake (\( p = .031 \) by two-way ANOVA) and of position (\( p < .0001 \)) were highly significant. There was a significant interaction between region and diet (\( p = .045 \)). Intestinal fructose transport per cm across intestinal regions was 51.6 ± 7.1 and 32.4 ± 4.1 nmol/min for CR1 and CRAL mice, respectively. One-way ANOVA indicated that fructose uptake in the CRAL was significantly (\( p < .02 \)) lower than that of CR mice in the proximal small intestine only.

Three days of AL feeding did not significantly influence D-fructose transport expressed per mg (\( p = .20 \) by two-way ANOVA) or per cm (\( p = .79 \)), whereas the effect of position was always highly significant (\( p < .0001 \), both as expressed per mg and per cm) (Table 2). Interactions between independent variables were not significantly different either per mg (\( p = .43 \)) or per cm (\( p = .92 \)).

**Total intestinal uptake capacity.** — Because uptake per mg and per cm were much lower in CRAL mice but intestinal weights and lengths were similar between CR1 and

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**Table 2. Uptake of D-Glucose, D-Fructose, and L-Proline in ACRAL Mice and Their Controls (CR2)**

<table>
<thead>
<tr>
<th>INTESTINAL REGION</th>
<th>D-Glucose</th>
<th></th>
<th></th>
<th>D-Fructose</th>
<th></th>
<th></th>
<th>L-Proline</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prox</td>
<td>Mid</td>
<td>Dis</td>
<td>Prox</td>
<td>Mid</td>
<td>Dis</td>
<td>Prox</td>
<td>Mid</td>
</tr>
<tr>
<td>CR2 (nmol/mg·min)</td>
<td></td>
<td>5.79 ± 1.28</td>
<td>6.80 ± 1.32</td>
<td>1.30 ± .49</td>
<td>1.62 ± .24</td>
<td>.76 ± .15</td>
<td>.37 ± .16</td>
<td>5.88 ± .81</td>
<td>6.45 ± 1.05</td>
</tr>
<tr>
<td>(nmol/cm·min)</td>
<td></td>
<td>224.5 ± 53.5</td>
<td>294.3 ± 73.2</td>
<td>18.6 ± 4.9</td>
<td>52.3 ± 11.2</td>
<td>28.4 ± 7.8</td>
<td>4.4 ± 0.8</td>
<td>177.4 ± 16.1</td>
<td>216.6 ± 31.6</td>
</tr>
<tr>
<td>ACRAL</td>
<td></td>
<td>5.86 ± .30</td>
<td>5.62 ± .70</td>
<td>1.09 ± .13</td>
<td>1.20 ± .24</td>
<td>.77 ± .10</td>
<td>.21 ± .05</td>
<td>5.93 ± .99</td>
<td>7.43 ± .84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>292.6 ± 26.0</td>
<td>231.4 ± 31.3</td>
<td>26.7 ± 5.8</td>
<td>47.6 ± 7.5</td>
<td>29.1 ± 4.8</td>
<td>3.6 ± 0.9</td>
<td>235.8 ± 40.9</td>
<td>282.2 ± 32.2</td>
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<tr>
<td>p-value</td>
<td></td>
<td>.95</td>
<td>.42</td>
<td>.65</td>
<td>.14</td>
<td>.89</td>
<td>.24</td>
<td>.97</td>
<td>.48</td>
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<tr>
<td>CR2 (nmol/mg·min)</td>
<td></td>
<td>224.5 ± 53.5</td>
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</tr>
<tr>
<td>(nmol/cm·min)</td>
<td></td>
<td>292.6 ± 26.0</td>
<td>231.4 ± 31.3</td>
<td>26.7 ± 5.8</td>
<td>47.6 ± 7.5</td>
<td>29.1 ± 4.8</td>
<td>3.6 ± 0.9</td>
<td>235.8 ± 40.9</td>
<td>282.2 ± 32.2</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>.24</td>
<td>.41</td>
<td>.32</td>
<td>.72</td>
<td>.93</td>
<td>.77</td>
<td>.12</td>
<td>.16</td>
</tr>
</tbody>
</table>

*Notes:* Values are means ± SE (n = 6–9); p-values are by one-way ANOVA. For CR2 and ACRAL definition, see Table 1. Prox, Mid, and Dis are proximal, middle, and distal small intestine, respectively. None of the differences was statistically significant.
CRAL mice, it follows that the total intestinal uptake capacities for D-glucose, D-fructose, and L-proline were each significantly lower in CRAL mice (Table 3). Because uptake per mg and per cm (Table 2) and intestinal weight and lengths were similar, total intestinal uptake capacity for these nutrients were also similar between CR2 and ACRAL mice.

Mucosal permeability to L-glucose. — Mucosal permeability as determined by L-glucose uptake was unaffected by the switch in caloric intake. Values for L-glucose uptake per mg were \( 0.99 \pm 0.21 \) (\( n = 9 \)) and \( 1.1 \pm 0.25 \) (\( n = 8 \)) pmol/min for CRAL and CR1 mice, respectively (no significant difference, \( p = .78 \)), whereas values for L-glucose uptake per cm were \( 27.3 \pm 6.8 \) (\( n = 9 \)) and \( 32.8 \pm 6.4 \) (\( n = 8 \)) pmol/min for CRAL and CR1 mice, respectively (no significant difference, \( p = .57 \)).

Transport of Amino Acids

L-proline. — We found a highly significant effect of the dietary switch to AL feeding for 1 mo (\( p = .0002 \) by two-way ANOVA) but not of intestinal position (\( p = .66 \)) on L-proline uptake per mg (Figure 3A). There was no significant interaction between intestinal position and diet (\( p = .97 \)). Intestinal proline transport per mg across intestinal regions was \( 7.72 \pm 0.48 \) and \( 4.80 \pm 0.51 \) nmol/min for CR1 and CRAL mice, respectively.

When L-proline uptake was expressed per cm, both diet switch (\( p = .004 \)) and intestinal region (\( p < .001 \)) had a significant effect on uptake (Figure 3B). However, there was no significant interaction between diet and region (\( p = .74 \)). Intestinal proline transport per cm across intestinal regions was \( 263 \pm 24 \) and \( 183 \pm 20 \) nmol/min for CR1 and CRAL mice, respectively.

Three days of AL feeding did not significantly influence L-proline transport expressed per mg (\( p = .42 \) by two-way ANOVA) or per cm (\( p = .06 \)); the effect of position on uptake per mg was not significant (\( p = .21 \)), but was highly significant (\( p = .0002 \)) on uptake per cm (Table 2). Interactions between independent variables were not significant either per mg (\( p = .82 \)) or per cm (\( p = .93 \)).

L-glutamine and L-aspartate. — There was a significant effect of diet on transport per mg of both L-glutamine (\( p = .027 \) by one-way ANOVA) (Figure 4A) and L-aspartate (\( p = .009 \)) (Figure 4C) when expressed per cm, transport of both L-glutamine (Figure 4B) and L-aspartate (Figure 4D) was still lower in the CRAL than in the CR1 mice, but differences were not significant (\( p = .12 \) and .08, respectively).

The 3-d switch in caloric intake did not significantly influence either L-glutamine or L-aspartate transport expressed per mg (\( p = .74 \) and .41, respectively) or per cm (\( p = .83 \) and .59, respectively). For L-glutamine, uptake per mg was \( 6.40 \pm 1.35 \) and \( 7.25 \pm 2.26 \) nmol/min, whereas uptake per cm was \( 212 \pm 74 \) and \( 235 \pm 58 \) nmol/min for ACRAL and CR2 mice, respectively. For L-aspartate, uptake per mg was \( 6.45 \pm 0.54 \) and \( 7.34 \pm .90 \) nmol/min while uptake per cm was \( 179 \pm 20 \) and \( 201 \pm 31 \) nmol/min for ACRAL and CR2 mice, respectively.

Table 3. Total Intestinal Capacity for D-Glucose, D-Fructose, and L-Proline

<table>
<thead>
<tr>
<th></th>
<th>D-Glucose</th>
<th>D-Fructose</th>
<th>L-Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1</td>
<td>14800 ± 2500</td>
<td>2300 ± 250</td>
<td>12500 ± 1400</td>
</tr>
<tr>
<td>CRAL</td>
<td>8320 ± 1260</td>
<td>1630 ± 110</td>
<td>9040 ± 720</td>
</tr>
<tr>
<td>p-value</td>
<td>.04</td>
<td>.04</td>
<td>.05</td>
</tr>
<tr>
<td>CR2</td>
<td>9730 ± 1620</td>
<td>1280 ± 180</td>
<td>7600 ± 760</td>
</tr>
<tr>
<td>ACRAL</td>
<td>8820 ± 1000</td>
<td>1220 ± 110</td>
<td>10700 ± 1390</td>
</tr>
<tr>
<td>p-value</td>
<td>.65</td>
<td>.79</td>
<td>.080</td>
</tr>
</tbody>
</table>

Notes: All capacity data are in nmol/min. Values are means ± SE (n = 6) and were determined by integrating uptake per cm along the length of the small intestine. For CR1, CR2, CRAL, and ACRAL data, see Table 1.
**L-alanine, L-leucine, and L-lysine.** — Transport of L-alanine, L-leucine, and L-lysine per mg or per cm was independent of the diet switch from chronic CR to AL feeding (Table 4). A 3-d switch in caloric intake also did not significantly influence transport expressed per mg or per cm (Table 5).

**Expression of Intestinal Sugar Transporters**

The specific PCR primers of mouse SGLT1, GLUT2, GLUT5, and GAPDH used in the PCR amplification generated 908-, 487-, 617-, and 306-bp products, respectively (Figure 5) as would be expected from the primer design. Steady-state levels of mouse GAPDH (Figure 5A), GLUT2 (Figure 5B), SGLT1 (Figure 5C), and GLUT5 (Figure 5D) showed no significant differences in expression according to age, caloric intake, and intestinal position ($p > .50$ for all enzymes and for each condition; Figure 6). Similar results were obtained in a Northern blot analysis using poly(A)$^+$ RNA pooled from the above samples. Three subsequent Northern blot analyses comparing transporter mRNA expression between 6- and 24-mo-old mice also showed levels of SGLT1, GLUT2, and GLUT5 mRNA to each be independent of age (results not shown).

**Table 4. Effect of a Switch From Chronic CR to 1 mo of AL Feeding on the Intestinal Transport of Alanine, Leucine, and Lysine in Aged Mice**

<table>
<thead>
<tr>
<th></th>
<th>L-Alanine</th>
<th>L-Leucine</th>
<th>L-Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1</td>
<td>8.84 ± .69</td>
<td>8.70 ± 1.28</td>
<td>3.66 ± .30</td>
</tr>
<tr>
<td>CRAL</td>
<td>8.66 ± .52</td>
<td>7.26 ± .96</td>
<td>2.86 ± .33</td>
</tr>
<tr>
<td>$p$-value</td>
<td>.84</td>
<td>.40</td>
<td>.092</td>
</tr>
<tr>
<td>CR1</td>
<td>284 ± 31</td>
<td>266 ± 34</td>
<td>91 ± 7</td>
</tr>
<tr>
<td>CRAL</td>
<td>291 ± 22</td>
<td>244 ± 35</td>
<td>90 ± 12</td>
</tr>
<tr>
<td>$p$-value</td>
<td>.85</td>
<td>.66</td>
<td>.933</td>
</tr>
</tbody>
</table>

*Notes: Values are means ± SE (n = 8-9). For CR1 and CRAL, see Table 1.*

**Table 5. Effect of a Switch From Chronic CR to AL Feeding for 3 d on the Intestinal Transport of Alanine, Leucine, and Lysine in Aged Mice**

<table>
<thead>
<tr>
<th></th>
<th>L-Alanine</th>
<th>L-Leucine</th>
<th>L-Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR2</td>
<td>7.87 ± .69</td>
<td>6.82 ± 1.09</td>
<td>3.76 ± .44</td>
</tr>
<tr>
<td>ACRAL</td>
<td>7.97 ± .79</td>
<td>6.86 ± 1.40</td>
<td>2.87 ± .54</td>
</tr>
<tr>
<td>$p$-value</td>
<td>.93</td>
<td>.99</td>
<td>.24</td>
</tr>
<tr>
<td>CR2</td>
<td>197 ± 31</td>
<td>245 ± 42</td>
<td>118 ± 21</td>
</tr>
<tr>
<td>ACRAL</td>
<td>244 ± 44</td>
<td>237 ± 49</td>
<td>120 ± 17</td>
</tr>
<tr>
<td>$p$-value</td>
<td>.47</td>
<td>.91</td>
<td>.94</td>
</tr>
</tbody>
</table>

*Notes: Values are means ± SE (n = 8-9). For CR2 and ACRAL data, see Table 1.*

**DISCUSSION**

We have recently shown that intestinal nutrient uptake is much greater in aged mice that had been chronically calorie restricted since 4 mo of age compared to that in same age AL controls (Casirola et al., 1996). When aged, AL mice are switched to CR, nutrient uptake rates do not increase even after 4 wk of CR. The main finding of this study extends...
Mechanisms of Intestinal Adaptation to CR

There are no nonspecific, diet-related changes in membrane permeability or in intestinal mass, each of which would result in parallel changes in uptake rates for different nutrients. Changes in enterocyte migration rate and in enterocyte lifetime may affect the maturity of cells lining the villus core and indirectly affect uptake because transporter number is greater in mature enterocytes (Ferraris and Diamond, 1997). Although the rates of enterocyte renewal and migration along the crypt–villus axis are not known during chronic CR, calorie restriction for 28 d reduces cell proliferation rates by 25–35% in the duodenum and jejunum (Lok et al., 1988) and perhaps extends cell lifetime from 55 to 72 h in the jejunum of aged mice (Ferraris and Vinnakota, 1995). Chronic CR may enhance transport by reducing cell migration rates and increasing the percentage of mature cells along the villi, but this type of mechanism is not specific for any type of nutrients, and we observe a specific response to CR.

The adaptive mechanism to chronic CR is specific to affected transporters of nonessential nutrients (nutrients that can be synthesized by biochemical pathways) because transport of essential amino acid lysine and leucine does not change. The absence of apparent changes in steady-state levels of SGLT1, GLUT2, and GLUT5 mRNA indicates that specific changes in transporter mRNA expression are not involved in long-term adaptive changes that occur with age and CR. This is not surprising; in sheep (Shirazi-Bechey et al., 1991) and rabbit (Smith et al., 1992) small intestine, alterations in levels of SGLT1 mRNA are also not correlated with changes in rates of Na⁺-dependent glucose uptake. Mammals that hibernate and undergo seasonal fasting also do not exhibit significant changes in levels of SGLT1 mRNA but do exhibit changes in intestinal glucose uptake (Carey, 1993). On the other hand, we have always found marked changes in levels of GLUT5 mRNA to be tightly linked with changes in facilitated fructose uptake rate in small intestine (Monteiro and Ferraris, 1997; Shu et al., 1997).

A specific mechanism commonly observed to underlie adaptive changes in nutrient transport is a change in number of nutrient transporters per cell or intestinal mucosa. For example, changes in phlorizin (a competitive inhibitor of intestinal glucose transport) binding explain most of the specific changes in glucose uptake that occur with diet and age (Ferraris and Diamond, 1997). This may be the main mechanism underlying adaptations to chronic CR because changes in uptake rate per mg intestine underlie all of the changes in nutrient uptake.

Intestinal Uptake Capacity

These dramatic increases in intestinal transport suggest that intestinal adaptations are an important component in an animal's adaptation to CR. To highlight the relevance of this adaptation to the whole animal and because mammalian metabolism changes as W^0.9, transport results can

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**Figure 6.** Average scanning density levels (relative to that in the proximal small intestine of 6-mo-old mice fed AL) of transporter mRNA levels (n = 3). Lanes: 1 = proximal small intestine from 6-mo-old mice fed AL; 2 = distal; 3 = proximal small intestine from 6-mo-old CR mice; 4 = distal; 5 = proximal small intestine from 24-mo-old mice fed AL; 6 = distal; 7 = proximal small intestine from 244no-old mice fed CR; 8 = distal; 9 = proximal small intestine from >30-mo-old mice fed AL; 10 = distal; 11 = proximal small intestine from >30-mo-old mice fed CR; 12 = distal.
also be analyzed by expressing total intestinal uptake capacity for a nutrient as a function of the metabolic mass of the mouse, according to the equation

$$\frac{\Sigma j}{W^{0.75}} = \left(\frac{X}{W^{0.75}}\right) \left(\frac{\Sigma j}{X}\right)$$

(Karasov et al., 1985, and Ferraris et al., 1989), where $\Sigma j$ is the total absorptive capacity of the small intestine; $W^{0.75}$ is the metabolic live mass ($W$ is body weight); $X$ is an anatomical measure of the small intestine (in this case, total intestinal weight); and $\Sigma j/W^{0.75}$ is total intestinal transport normalized to metabolic live mass. This last expression allows us to better compare total absorption in mice with different body weights, such as CRAL and CR1. $X/W^{0.75}$ is the anatomical factor and $\Sigma j/X$ the physiological factor contributing in absorptive capacity. Using the body and intestinal weights from Table 1 and total absorptive capacities for D-glucose, D-fructose, and L-proline from Table 3, we find that for each of these nutrients the anatomical factor varies very little between CRAL and CR1 mice or between CR2 and ACRAL mice. In fact, weight of the small intestine did not change significantly with CR in the three batches used in the present study and in the previous study (Casirola et al., 1996).

Decreases in total absorptive capacity as normalized to metabolic weight ($\Sigma j/W^{0.75}$) occurring during a switch to AL are instead tightly linked to decreases in the physiological factor, $\Sigma j/X$, suggesting that less of a nutrient is absorbed for the same amount of intestinal tissue in mice switched to AL feeding. For D-glucose, $\Sigma j/W^{0.75}$ of CRAL mice is about half that of CR1 mice, as is the $\Sigma j/X$ factor; for D-fructose and L-proline, $\Sigma j/W^{0.75}$ of the CRAL mice is 0.6 times that of the CR1 mice, and so is $\Sigma j/X$. Hence, the adaptive mechanism used by aged mice for changes in caloric intake is similar to that used for changes in levels of dietary carbohydrate or protein: a change in the number of transporters per mass of intestinal tissue (Ferraris and Diamond, 1997). This is not surprising because both conditions result in decreases in luminal concentrations of nutrients. The response to a change in dietary level, however, is rapid (=1 day). In contrast, the response to a change in caloric intake takes between 4 and 28 d, perhaps even longer (Cao and Ferraris, unpublished observations).

**Signals for Adaptation to CR**

The proximate signal for rapid increases in uptake rates during increases in levels of dietary carbohydrate or protein is mainly an increase in luminal concentration of nutrients which are substrates of transporters (Ferraris and Diamond, 1997; Shu et al., 1997). In chronic CR, there is a chronic reduction in total luminal contents associated, paradoxically, with increases in rates of nutrient uptake after several months of CR (Casirola et al., 1996; Cao and Ferraris, unpublished observations). This increase in uptake induced by chronic CR cannot be induced by 4 wk of CR. When this chronic reduction in luminal content is instead abolished by AL feeding and luminal content increases abruptly, uptake decreases within 4 wk of AL feeding. This marked difference suggests a signal(s) for control of uptake rate by CR that is different from that by changes in levels of dietary nutrients. Clearly it is not luminal concentration of nutrients which should decrease with CR and increase with CRAL. The gradual stimulatory effect of CR on uptake suggests a type of regulatory control, perhaps a hormone, that reaches threshold only after several months of CR. When AL refeeding is reinstated and uptake decreases, perhaps this hormone drops below threshold as well.

The influence of caloric intake on intestinal absorption is not clear. In this study comparing aged mice consuming different amounts of food containing the same caloric density, there are no changes associated with the switch from CR to AL in membrane permeability or in intestinal mass, each of which would result in parallel changes in uptake rates for different nutrients. In contrast, intestinal glucose uptake is correlated with caloric density of food and intestinal mass in young adult rats consuming for several days similar amounts of food containing varying amounts of calories (Plasen et al., 1992; Dameto et al., 1994). This suggests that chronic consumption of a lesser amount of food activates different types of adaptive signals and mechanisms from consumption of same amounts of foods which vary in caloric density. There has been no study on the effect of this latter type of caloric restriction on life span.

Possible hormonal influences include that of glucagon, which is secreted at a higher rate during fasting in the rat (Potter and Morris, 1980) and decreases $K_c$ and increases $J_{\text{max}}$ of glucose transport in the rat. The increase in secretion of this hormone during fasting could be one of the possible factors in the increase of sugar uptake observed in CR; its action would cease with the switch to AL caloric intake. Another possible hormonal influence is the enhanced diurnal elevation of glucocorticoids during CR (Klebanov et al., 1995).

Changes in body weight are not correlated with changes in intestinal absorption, because body weight increases significantly before any decrease in absorption is observed in ACRAL mice (this study) and because body weight decreases significantly without any change in intestinal absorption in aged mice switched from AL to CR for 1 mo (Casirola et al., 1996).

**Caloric Intake Switch Affects Transport Rates of Nonessential Nutrients**

Why are transport rates of certain nutrients subject to changes in caloric intake while those of others are not? The adaptive response of amino acid transport to a diet switch was not univocal. Transport of proline, glutamine, and aspartate in senescent mice was significantly reduced by a switch from chronic CR to 4 wk of AL feeding. This finding is in keeping with a previous observation that transport of these same amino acids is significantly higher in chronically CR compared to that in AL-fed aged mice (Casirola et al., 1996). In contrast, transport of alanine, leucine, and lysine was not affected by the switch from chronic CR to AL feeding. Similarly, transport of these amino acids was the same between chronically CR or AL-fed mice (Casirola et al., 1996). Leucine and lysine are both essential amino acids, whereas alanine is absorbed in mouse intestine mainly by sharing the transporters (B and B°'; Ganapathy et al., 1994) for many other essential amino acids; hence, all three amino acids whose uptake remains independent of
caloric intake are those that are absorbed by transporters widely shared not only by essential amino acids but also by nonessential ones as well. In contrast, two amino acids whose uptakes clearly vary with caloric intake are absorbed by more specific transporters ferrying mainly nonessential amino acids: proline has its own carrier whereas aspartate is absorbed by a carrier mainly for the two acidic (and nonessential) amino acids (Karasov et al., 1986). The carrier for the polar amino acid glutamine is not clearly known for mice. Because glucose and fructose are also calorie-generating and nonessential nutrients, the type of regulation observed in CR may be similar to one described by Diamond and Karasov (1987) and later reviewed by Ferraris and Diamond (1997) for nutrients used mainly as energy source. In these reviews on dietary regulation of nutrient uptake, only the uptake of nonessential nutrients was observed to increase monotonically with increasing concentrations of that nutrient in the diet. Uptake of essential, non-calorie-yielding nutrients such as vitamins or minerals decreased with increasing concentrations in the diet or body stores, whereas the uptake pattern of essential and calorie-yielding nutrients such as essential amino acids was a mixture of the two previous patterns: modestly increasing in uptake at high dietary concentrations, yet also increasing at very low concentrations.

There are four teleological reasons underlying these uptake patterns: (1) synthesis and maintenance costs, (2) caloric payoff, (3) fixed requirements, and (4) toxicity (Diamond and Karasov, 1987; Ferraris and Diamond, 1997). A transporter may be repressed if biosynthetic costs exceed the benefits a transporter provides, and those benefits can be calories for use as metabolic fuel or be an essential nutrient crucial in the metabolic process. Transporters for calorie-generating nutrients should be upregulated by their substrates because metabolizable nutrients yield calories in proportion to the amount absorbed. Animals that are chronically CR clearly benefit by enhancing the absorption rates of nutrients used mainly as calorie sources. Switching to an AL diet perhaps reduces the magnitude of the benefit, and absorption rates go down.

Unsolved Problems and Future Work

Because we allowed ACRAL and CRAL mice free access to food, many would typically eat mostly at night. In contrast, we fed our CR mice between 10 am and 2 pm. Thus, the decrease in uptake with AL feeding may also be due to time of feeding. This disparity in time of feeding may not be critical because recent studies in our laboratory reversed the light cycle so that peak feeding time for CR and AL mice is now similar, and we still found dramatic differences in intestinal nutrient uptake between chronic CR and AL mice (Cao and Ferraris, unpublished observations).

The effect of CR on intestinal nutrient uptake seems clear, but the signals and mechanisms underlying this adaptation need to be identified. Because changes in uptake are independent of changes in steady-state levels of mRNA, perhaps posttranscriptional mechanisms are being utilized. If enhancement of intestinal uptake by chronic CR in aged mice is reversed by 1 mo of AL feeding, and if 1 mo of CR does not enhance uptake in aged AL mice, there may be different signals and/or mechanisms of adaptation from AL to CR, and for CR to AL. Understanding these signals and mechanisms would increase our understanding of metabolic adaptations to CR.

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References


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