

Calories and aging alter gene expression for gluconeogenic, glycolytic, and nitrogen-metabolizing enzymes

JOSEPH M. DHAHBI,¹ PATRICIA L. MOTE,¹ JOHN WINGO,¹ JOHN B. TILLMAN,¹ ROY L. WALFORD,² AND STEPHEN R. SPINDLER¹

¹Department of Biochemistry, University of California, Riverside 92521;

and ²Department of Pathology, School of Medicine, University of California, Los Angeles, California 90024

Dhahbi, Joseph M., Patricia L. Mote, John Wingo, John B. Tillman, Roy L. Walford, and Stephen R. Spindler. Calories and aging alter gene expression for gluconeogenic, glycolytic, and nitrogen-metabolizing enzymes. *Am. J. Physiol.* 277 (Endocrinol. Metab. 40): E352–E360, 1999.—We characterized the effects of calorie restriction (CR) on the expression of key glycolytic, gluconeogenic, and nitrogen-metabolizing enzymes in mice. Of the gluconeogenic enzymes investigated, liver glucose-6-phosphatase mRNA increased 1.7- and 2.3-fold in young and old CR mice. Phosphoenolpyruvate carboxykinase mRNA and activity increased 2.5- and 1.7-fold in old CR mice. Of the key glycolytic enzymes, pyruvate kinase mRNA and activity decreased ~60% in CR mice. Hepatic phosphofructokinase-1 and pyruvate dehydrogenase mRNA decreased 10–20% in CR mice. Of the genes that detoxify ammonia generated from protein catabolism, hepatic glutaminase, carbamyl phosphate synthase I, and tyrosine aminotransferase mRNAs increased 2.4-, 1.8-, and 1.8-fold with CR, respectively. Muscle glutamine synthetase mRNA increased 1.3- and 2.1-fold in young and old CR mice. Hepatic glutamine synthetase mRNA and activity each decreased 38% in CR mice. These CR-induced changes are consistent with other studies suggesting that CR may decrease enzymatic capacity for glycolysis and increase the enzymatic capacity for hepatic gluconeogenesis and the disposal of byproducts of muscle protein catabolism.

calorie restriction; enzyme activity; metabolism; mice; mRNA

DIETARY CALORIE RESTRICTION (CR) delays most age-related physiological changes, is the only method known for extending life span in homeothermic vertebrates, and is the most effective means known for reducing cancer incidence and increasing the mean age of onset of age-related diseases and tumors (31, 32). The effects of CR are robust; they have been reported in every species tested (32). Although many of the physiological consequences of CR were first described more than sixty years ago, no consensus has yet emerged regarding its mode of action.

In rodents, primates, and humans, CR reduces 24-h and fasting blood glucose and insulin concentrations, as well as maximum glucose and insulin concentrations during oral glucose tolerance tests (5, 10, 28). Several kinds of evidence support a role for glucose and insulin in many of the pathologies of aging. For example, chronic hyperglycemia is associated with long-term

neurological complications, microvascular disorders, basement membrane thickening, and impaired cellular immunity (19). Hyperinsulinemia is associated with coronary heart disease, hypertension, and atherosclerosis (24). All of the pathologies associated with elevated glucose levels are reduced or mitigated entirely by CR. Whether glucose and insulin have a role in determining the rate of aging itself is unknown. However, there is evidence suggesting that it may.

There is only one multicellular organism for which life span-regulating gene systems have been partially elucidated at the molecular level. In *Caenorhabditis elegans*, insulin receptor signaling appears to have a central role in aging. The insulin receptor homologue of *C. elegans*, daf-2, acts on daf-16, a hepatocyte nuclear factor 3 (HNF-3)/forkhead transcription factor family member, to alter energy metabolism and development (8). In mammals, insulin may also mediate some of its actions by altering the activity of HNF-3 (16). Three of the four well-studied insulin-responsive genes, phosphoenolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT), and insulin-like growth factor binding protein-1, appear to be regulated by HNF-3 through insulin-response sequences that also are binding sites for HNF-3 (16).

Thus changes in carbohydrate metabolism may have an important role in the anti-aging effects of CR. To examine this hypothesis, the mRNA and in some cases the activities of key glycolytic, gluconeogenic, and nitrogen-metabolizing enzymes were quantified in hepatic and extrahepatic tissues of CR and control mice. Our studies show that CR leads to an increase in the mRNA and/or activity of key enzymes of hepatic gluconeogenesis, decreases the mRNA and/or activity of key enzymes of hepatic glycolysis, and increases the mRNA and/or activity of key enzymes responsible for the disposal of nitrogen derived from muscle protein catabolism for energy production. These CR effects oppose age-related changes in the mRNA and/or activity of many of these key metabolic enzymes. The results are consistent with studies in the literature suggesting that CR enhances the turnover of extrahepatic protein, extending higher levels of protein turnover into old age.

MATERIALS AND METHODS

Mice. Females of the long-lived F₁ hybrid strain C3B10RF₁ were used (22). This is the longest-lived strain of which we are aware, and one we have used extensively in our studies. Use of an F₁ hybrid avoids the possible effects of inbreeding-related genetic problems on the data. Mice were maintained as previously described (2). There were eight mice in each age

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and dietary group. To conserve tissue, not all of these animals were used in each experiment shown. The same six mice from each group were used in all experiments, with two exceptions. In the pyruvate kinase (PK) activity assays, only four of the six mice in each group were utilized. In the PEPCK activity assays, all eight mice were used. At the time of use, the old CR and control mice weighed 28.9 ± 2.9 and 49.9 ± 5.3 g, respectively, and the young CR and control mice weighed 20.8 ± 2.1 and 24.9 ± 3.4 g, respectively. Young mice were 7 mo old, and old mice were 28 mo old.

Diets. Mice were weaned at 28 days, housed individually, and subjected to one of the two dietary regimens described below. The composition of the defined diets and feeding regimen has been described in detail (20). They are formulated so that dietary groups receive approximately equal amounts of protein, corn oil, minerals, and vitamins per gram of body weight. The amount of carbohydrates varied between groups. Control mice consumed ~ 105 kcal (~ 449 kJ) each week. This is approximately the amount of food required to support normal growth of mice (25). The control mice were fed daily Monday through Friday. The 50% CR mice consumed ~ 52 kcal (225 kJ) per wk and were fed Mondays, Wednesdays, and Fridays. On Fridays, both groups were given a 3-day allotment of food. Mice were fed at 0900. Mice had free access to water. For the studies, mice were fed a normal allotment of food Monday morning, and all the food was eaten within 45 min. They were fasted for ~ 23 h and were killed on Tuesday morning.

Nucleic acid hybridization probes. A mouse phosphofructokinase-1 (PFK-1) 1-kb cDNA probe was excised with *EcoR* I and *Xho* I from American Type Culture Collection (ATCC, Rockville, MD) plasmid no. 974547. A 1.4-kb fragment corresponding to the 3' end of the cDNA of rat PKF-2/fructose-2,6-bisphosphatase 2 (FBPase-2) was produced by *EcoR* I digestion of pEMBL18. The 650-bp *EcoR* I fragment corresponding to the 3' end of the rat fructose-1,6-bisphosphatase (Fru 1,6-P₂ase) cDNA was excised from pEMBL18. Both PKF-2/FBPase-2 and Fru 1,6-P₂ase cDNAs were a gift from Dr. M. R. El-Maghrabi (State University of New York, Stony Brook, NY). Mouse glucokinase (GK) cDNA corresponding to exons 2–7 was excised with *Bam*H I and *EcoR* I from mGK2–7 (a gift from Dr. L. Chan, Baylor College of Medicine, Houston, TX). A 1070-bp fragment from nucleotides 730–1820 of murine glucose-6-phosphatase (G-6-Pase) was excised with *EcoR* I from pWT28 (a gift from Dr. J. Y. Chou, Department of Health and Human Services, Bethesda, MD). The rat glutaminase 1.4-kb cDNA fragment was excised with *EcoR* I from pGLN2.0 (a gift from Dr. M. Watford, The State University of New Jersey, New Brunswick, NJ). The rat CPSI probe was a 1.2-kb *Pst* I and *EcoR* I fragment excised from pHN3491974547 (ATCC). The mouse pyruvate dehydrogenase (PDH) 2.8-kb cDNA fragment was excised with *EcoR* I from pmIEI- α (a gift from Dr. H. Dahl, The Murdoch Institute, Melbourne, Australia). The 1.3-kb PEPCK coding fragment was produced by *Sph* I followed by *Sal* I digestions of pGEM5ZF (a gift from Dr. D. K. Granner, Vanderbilt University School of Medicine, Nashville, TN). Mouse TAT and glutamine synthetase (GS) cDNA were synthesized with Ready-To-Go PCR beads (Pharmacia Biotech, Piscataway, NJ) by use of murine liver cDNA. The primers for TAT were complementary to bases 289 to 320 for the forward primer and bases 1136 to 1166 for the return primer (Genbank Accession no. X02741). Primers for GS were complementary to bases 731 to 760 for the forward primer and bases 1889 to 1918 for the return primer (Genbank Accession no. X16314). A murine muscle PK (MPK) cDNA fragment of 1908 bp was excised with *EcoR* I from mPKm2 (a gift from Dr. H. Ariga,

Hokkaido University, Sapporo, Japan). Liver PK (LPK) cDNA was synthesized from the cDNA library described above, using primers complementary to bases 583 to 610 for the forward primer and bases 1767 to 1795 for the return (Genbank Accession no. D63764). These primers were in regions of low homology between the murine LPK and MPK cDNAs (7). Multiprime labeling of each of the PK cDNAs and probing of liver and muscle RNA Northern blots showed that there was little if any cross-hybridization of the muscle probe with LPK mRNA or of the liver probe with the MPK mRNA. All of the blots probed with the PK cDNAs received a final wash in $0.1 \times$ saline sodium citrate (SSC) at 55°C for 30 min (15).

RNA isolation and visualization. Mice were killed by cervical dislocation, and tissues were rapidly removed. Muscle from the hind legs and back was removed and pooled for each animal. Tissues were flash-frozen in liquid nitrogen. Approximately 0.2 g of frozen liver tissue was homogenized for 40 s in 4 ml of TRI Reagent (Molecular Research Center, Cincinnati, OH) by use of a Tekmar Tissueemizer (Tekmar, Cincinnati, OH) at a setting of 55. RNA was isolated as described by the TRI Reagent supplier. RNA was resuspended in FORMAZOL (Molecular Research Center), and Northern and dot blots were performed using 20 and 10 μg of RNA, respectively. The RNA was analyzed using Northern blots to verify its integrity, and specific mRNA levels were quantified using dot blots. cDNAs were radioactively labeled with [α -³²P]dCTP to a specific activity of 1.7 MBq/ μg by multiprime labeling with a kit according to the method of the supplier (Pharmacia, Piscataway, NJ). An oligonucleotide complementary to 18S rRNA was 5' end-labeled with [γ -³²P]ATP and T₄ polynucleotide kinase (New England Biolabs, Beverly, MA) to a specific activity of 7 KBq/pmol. Northern and dot blots were hybridized with 22 and 4.2 KBq/ml of the probes for specific mRNA and 18S rRNA, respectively. Hybridization was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Specific mRNA levels were normalized to the level of S-II transcription factor mRNA. S-II and 18S mRNAs are not affected by diet or age (14, 21).

Stability of specific mRNA. Intraperitoneal injections of 4 mg/kg body weight actinomycin D (Sigma, St. Louis, MO) in PBS were administered to mice. Sham-injected controls received identical injections of the PBS vehicle. After 6, 12, and 18 h, livers were removed and RNA was prepared as described in *RNA isolation and visualization*. Specific mRNA levels were determined using dot and Northern blots normalized to the level of 18S rRNA, because rRNA is much more stable than mRNA, and its level does not change with diet (14). Three 24-mo-old mice from each dietary group were used for each time point. The control fed mice were 41.6 ± 5.8 g and the CR mice were 27.2 ± 2.5 g. Half-lives were determined by inspection of semilogarithmic plots of the data. All semilogarithmic plots were linear.

Enzyme assays. Approximately 30 mg of tissue were sonicated (Branson model 350 Sonifier Cell Disrupter) in 300 μl of the appropriate homogenization buffer (in mM: 50 Tris, pH 7.4, 1 MnCl_2 , and 2 dithiothreitol for PEPCK; 100 Tris, pH 7.5, 10 MgSO_4 , 150 KCl, and 0.2 EDTA for LPK; 150 potassium phosphate, pH 7.3, 0.2 EDTA, and 0.5% Triton X-100 for MPK; and 50 imidazole, pH 7.0, 0.2 EDTA, 2 β -mercaptoethanol, and 0.5% Triton-X 100 for GS). PEPCK activity was assayed and units were calculated as described (17). The final reaction mixture contained 50 mM Tris, pH 7.4, 1 mM MnCl_2 , 20 mM sodium bicarbonate, 0.5 mM phosphoenolpyruvate (PEP), 0.1 mM NADH, 0.1 mM dGDP, rotenone (2 $\mu\text{g}/\text{ml}$; Sigma), and malate dehydrogenase (2 units/ml; Boehringer). Reactions were initiated by addition of 500 μg of the liver homogenate to 300 μl of reaction mixture in a final volume of

600 μ l. PK activity was measured by modification of the technique of Imamura and Tanaka (6). The final reaction mixture contained 100 mM Tris, pH 7.5, 10 mM MgSO_4 , 150 mM KCl, 2 mM PEP, 2 mM ADP, 170 μ M NADH, 0.5 mM Fru 1,6- P_2 ase, and 4 units/ml of lactate dehydrogenase. Reactions were initiated by addition of 150 μ g protein of the liver homogenate or 10 μ g protein of the muscle homogenate to 400 μ l of reaction mixture in a final volume of 800 μ l. The change in absorbance at 340 nm was determined between minutes 1 and 2. The units were calculated as described (6) using a conversion factor of 0.80 A_{340} units equals 100 μ g/ml NADH (Sigma). GS activity was determined by measuring the conversion of ^{14}C glutamate (Amersham, England) to ^{14}C glutamine as described (18). The reaction buffer contained 2.5 mM ^{14}C glutamate (0.5 Ci/mol), 7.5 mM ATP, 30 mM MgCl_2 , 25 mM NH_4Cl , and 50 mM imidazole/HCl buffer, pH 7.0. Reactions were initiated by addition of 20 μ g of the liver homogenate protein or 300 μ g of the muscle homogenate protein to 100 μ l of reaction mixture in a final volume of 200 μ l. After incubation for 15 min at 37°C, the reactions were stopped by adding 1 ml of ice-cold 20 mM imidazole/HCl buffer, pH 7.0. A 300- μ l aliquot was then loaded on a 1.2-ml column of AG 1-X8 (chloride form) anion exchange resin (Bio-Rad) and washed with 1.5 ml imidazole/HCl buffer, pH 7.0. The ^{14}C glutamine contained in the effluent was counted by liquid scintillation.

Glycogen determination. Liver glycogen content was determined as previously described (1). Briefly, 40- to 50-mg aliquots of liver were sonicated to homogeneity in three volumes of 30% KOH and heated to 100°C for 30 min. The homogenates were cooled to room temperature, and ethanol was added to a final concentration of 66%. After overnight precipitation at 4°C, centrifugation at 3,000 g , and removal of supernatant, the pellet was resuspended in 200 μ l of 0.6 N NaOH. This suspension was heated to 100°C for 2 h, neutralized, and centrifuged to remove debris. Ten microliters of this solution were assayed for glucose content in a 0.5-ml reaction by use of a Glucose (HK) Assay 10 Kit (Sigma).

Statistical analysis. Student's unpaired t -test was used to analyze the effect of CR on hepatic PEPCK and PK activity levels in the 28-mo-old animals. The effects of age, diet, and their interaction on specific mRNA and activity levels were analyzed using a two-factor ANOVA with age and diet as the factors. There was no significant difference in the age \times diet interaction between control and CR mice. The significance of differences between any two groups was tested by Tukey's pairwise comparisons. When the means were significantly different, an "a" was assigned to the lowest value. If a value was significantly different from that of group "a," it was categorized "b." Groups that were not significantly different from "a" or "b" were labeled "ab." ANOVA computations were performed using Minitab statistical software (13). A 95% level of confidence was considered significant.

RESULTS

We have investigated the hypothesis that CR alters the expression of key gluconeogenic, glycolytic, and nitrogen-metabolizing enzymes. The level of mRNA and, in many cases, the activity of these enzymes were determined.

Hepatic gluconeogenic enzymes. PEPCK catalyzes the first committed step of gluconeogenesis in the liver (Fig. 1). Hepatic PEPCK activity was 1.7-fold higher in old CR mice than in control mice ($P = 0.0005$; Fig. 2B). The abundance of PEPCK mRNA was 1.9-fold and

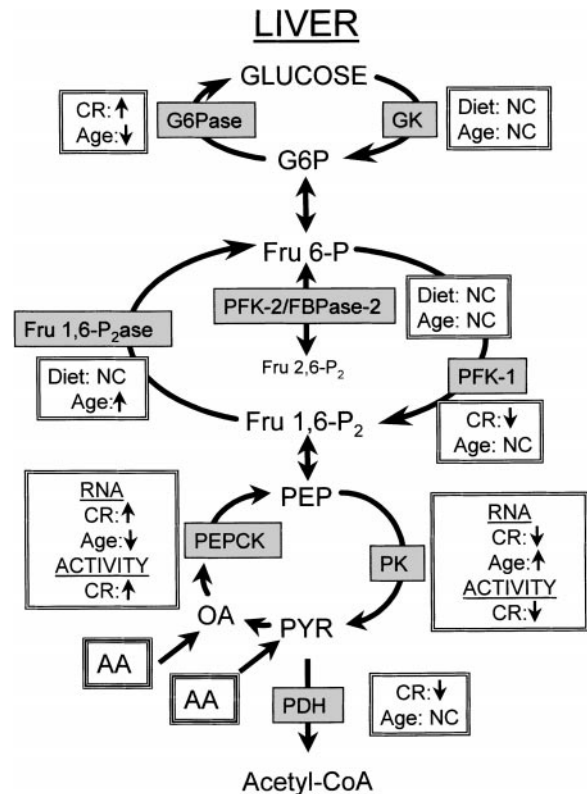


Fig. 1. Summary of effects of age and calorie restriction (CR) on glycolytic and gluconeogenic pathways of the liver. Glycolytic metabolism in the liver involves three irreversible, regulated steps. Glucokinase (GK) initiates glucose metabolism by phosphorylation of C-6, yielding glucose 6-phosphate (G-6-P). The committed step in glycolysis, and the second irreversible and regulated step, is the phosphorylation of fructose 6-phosphate (Fru 6-P) by phosphofructokinase-1 (PFK-1) to produce fructose 1,6-bisphosphate (Fru 1,6-P₂). The third irreversible step controls the outflow of the pathway. Phosphoenolpyruvate (PEP) and ADP are utilized by pyruvate kinase (PK) to produce pyruvate (PYR) and ATP. Pyruvate dehydrogenase (PDH) oxidatively decarboxylates pyruvate to form acetyl-CoA. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the first committed step in gluconeogenesis. The main noncarbohydrate precursors for gluconeogenesis are amino acids from the diet and from muscle protein breakdown. Other organs also contribute amino acids, but muscle is the major source. Most of these amino acids are converted to oxaloacetate (OA), which is metabolized to PEP by PEPCK. In the second regulated and essentially irreversible step in gluconeogenesis, fructose 1,6-bisphosphatase (Fru 1,6-P₂ase) catalyzes the formation of Fru 6-P from Fru 1,6-P₂. Finally, in the third essentially irreversible reaction of gluconeogenesis, glucose is formed by the hydrolysis of G-6-P in a reaction catalyzed by glucose-6-phosphatase (G-6-Pase). Substrates are not boxed, enzyme names are in shaded boxes, summaries of experimental results are in double-bordered boxes, and amino acids are indicated (AA) in triple-bordered boxes. A down or up arrow indicates a general decrease or increase in expression of the gene in CR mice. Arrows after "Age" indicate general results for aged animals in both dietary groups. NC, no change.

2.2-fold greater in the liver of young and old CR mice, respectively, than it was in control mice of the same ages ($P < 0.0001$ and $P = 0.0001$; Fig. 2A). There was also a small age-related decrease in PEPCK mRNA in the liver ($P = 0.006$). The increase in PEPCK mRNA and activity is consistent with the idea that CR induces higher rates of gluconeogenesis in the livers of CR mice.

In the muscle, PEPCK is part of the alanine synthetic pathway. Alanine is derived from the degradation of

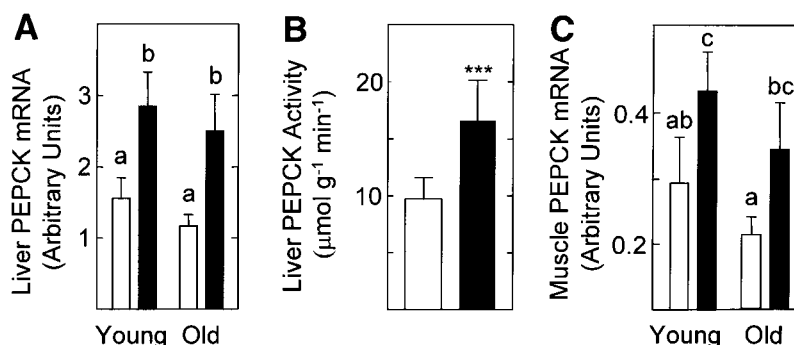


Fig. 2. Effects of age and diet on PEPCK mRNA and activity. Liver and muscle PEPCK mRNA and liver PEPCK activity were quantified. *A*: PEPCK mRNA abundance in liver of young and old, control (open bars) and CR (closed bars) mice (6 animals from each dietary regimen for each age group). *B*: liver PEPCK activity in old control and CR mice ($n = 8$ mice of each dietary group). Student's unpaired t -test was used to analyze the effect of CR on hepatic PEPCK in this group of mice. Results are very highly significant ($***P < 0.001$). *C*: PEPCK mRNA levels in mixed hind leg and back muscle of individual mice from group in *A*. Significance of differences between any 2 groups was tested by Tukey's pairwise comparisons. Bars labeled with different superscript letters are statistically significantly different ($P < 0.05$).

branched-chain amino acids, which are unique in that their degradation is mainly initiated in muscle rather than in liver. Newly synthesized alanine is transported to the liver to serve as a gluconeogenic precursor. PEPCK mRNA was increased in the muscle of both young and old CR mice (Fig. 2C; $P = 0.0045$ and $P = 0.0061$). This increase was similar to that found in their liver.

G-6-Pase produces glucose from glucose 6-phosphate (G-6-P), allowing its release from the liver into the circulation (Fig. 1). G-6-Pase mRNA was 1.6-fold and 2.3-fold higher in the liver of young and old CR mice than it was in control mice ($P = 0.0004$ and $P = 0.0015$; Fig. 3). These results are also consistent with the idea that there is an increase in hepatic gluconeogenesis in CR mice.

The level of Fru 1,6-P₂ase mRNA was unaffected by diet, although it did increase with age by ~25% in control and CR mice (Table 1; $P = 0.0005$ and $P = 0.0012$).

Liver glycogen. After 24 h without food, liver glycogen was depleted in both control and CR mice. Liver glycogen content was 0.80 ± 0.25 and 0.80 ± 0.18 mg/g liver in control and CR mice, respectively.

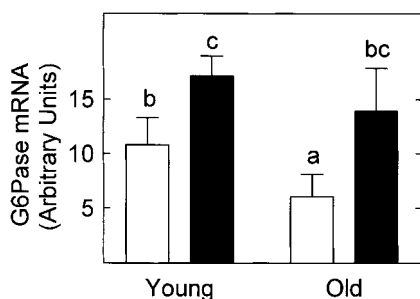


Fig. 3. Effects of age and diet on G-6-Pase mRNA levels in mouse liver. G-6-Pase mRNA levels in liver of young and old, control (open bars) and CR (closed bars) mice were determined. Results are means \pm SD; $n = 6$ mice for each dietary regimen and age group. Message levels were assessed by 2-factor ANOVA with age and diet as factors. Statistical analysis of effects of age and diet is shown as in Fig. 2.

Tissue specificity. The kidney is the second major site of gluconeogenesis. Age significantly decreased expression of both G-6-Pase and PEPCK mRNA in the kidney ($P = 0.001$ and $P = 0.009$; Table 2), as it does in the liver. However, unlike the liver, CR had no effect on the expression of G-6-Pase and PEPCK mRNA in the kidney ($P = 0.30$ and $P = 0.56$). Thus the effects of diet on the enzymatic capacity for gluconeogenesis are highly specific to the liver, whereas the age effect is present in both tissues.

Muscle nitrogen-metabolizing enzymes. Mice are in the postabsorptive state after 24 h of fasting. The major source of extrahepatic substrates for gluconeogenesis during fasting is amino acids derived from muscle (3). Muscle cells also utilize amino acids derived from protein turnover directly in the tricarboxylic acid cycle (Fig. 4). Muscle protein catabolism involves two steps collectively called a transamination reaction. Transamination leads to the liberation of the amino nitrogen as ammonia. Probably because of its extreme toxicity, this ammonia is rapidly transferred to glutamate by GS, producing glutamine. This is the only enzymatic reaction specific to the formation of glutamine in muscle (9).

CR increased muscle GS mRNA in old mice (2.1-fold; $P = 0.0008$; Fig. 5C). It also increased GS mRNA in young mice, although this increase did not reach statistical significance (1.3-fold; $P = 0.094$). There was an age-related 50% decrease in the expression of muscle GS mRNA in control mice ($P = 0.001$; Fig. 5C) but not in CR mice. Repeated attempts to quantify GS activity in muscle by use of two published techniques were unsuccessful. The muscle activity was ~50-fold below that of liver, and there were limited quantities of tissue available. However, CR clearly induced GS mRNA levels in muscle of old CR mice.

Hepatic nitrogen-metabolizing enzymes. We examined the expression of liver genes essential for the disposal of the nitrogen derived from amino acid catabolism. In the liver, glutamine produced in the muscle is metabolized by the enzyme glutaminase into glutamate

Table 1. *Hepatic abundance of GK, PDH, PFK-1, PFK-2/FBPase-2, and Fru 1,6-P₂ase mRNA in young and old, control and CR mice*

mRNA	Diet				2-Factor ANOVA P Values	
	Control		CR		Diet	Age
	Young	Old	Young	Old		
GK	1.03 ± 0.31	1.17 ± 0.13	0.96 ± 0.16	1.04 ± 0.28	0.32	0.24
PDH	11.43 ± 1.07 ^b	11.17 ± 1.18 ^b	10.63 ± 1.30 ^{ab}	8.88 ± 1.56 ^a	0.01	0.08
PFK-1	3.63 ± 0.56	4.03 ± 0.87	3.38 ± 0.16	3.20 ± 0.32	0.02	0.57
PFK-2/FBPase-2	2.00 ± 0.38	2.03 ± 0.25	1.92 ± 0.24	2.15 ± 0.45	0.88	0.35
Fru 1,6-P ₂ ase	1.67 ± 0.25 ^a	2.29 ± 0.16 ^b	1.68 ± 0.27 ^a	2.23 ± 0.15 ^b	0.80	<0.001

Values are means ± SD expressed in phosphorimager units normalized to S-II transcription factor; *n* = 6 mice. Mice 7 and 28 mo of age were fed control and calorie-restricted (CR) diets as described in MATERIALS AND METHODS. GK, glucokinase; PDH, pyruvate dehydrogenase; PFK-1, phosphofructokinase-1; PFK-2/FBPase-2, PFK-2/fructose-2,6-bisphosphatase-2; Fru 1,6-P₂ase, fructose 1,6-bisphosphatase. Significance of differences between any two groups was tested by Tukey's pairwise comparisons. Within a row, values with the same superscript letter are not significantly different (*P* > 0.05). Where no statistical difference exists between values in a row, no superscript letter is used. Analysis of the mRNA levels by 2-factor ANOVA showed no interactions between age and diet for any of the genes listed.

and ammonia (Fig. 4). The ammonia derived from this reaction can be returned to the glutamine pool by liver glutamine synthetase. However, CR significantly reduced GS activity in the liver of young (*P* < 0.0001) and old (*P* = 0.021) mice (Fig. 5B). A similar decrease in liver GS mRNA was found in young and old CR mice (*P* < 0.0001 and *P* = 0.0006, respectively; Fig. 5A). These changes were the opposite of those found in muscle (Fig. 5C). These results suggest that in CR mice the return of glutamate to the blood glutamine pool is reduced in the liver, likely making glutamate available as a substrate for hepatic gluconeogenesis (Fig. 4).

With age, there was a significant decrease in liver GS mRNA in control (*P* = 0.0005) but not CR (*P* = 0.15) mice. This age-related decrease is similar to that found in muscle, suggesting that in control mice, aging is accompanied by a decrease in the shuttling of nitrogen and carbon from the muscle to the liver through the glutamine-glutamate pathway. This decrease might result from the well-described decrease in muscle protein synthesis and degradation with age (27).

If the changes in GS mRNA and activity in CR mice are related to an increase in the mobilization of nitrogen and carbon by the muscle for export to the liver, one might expect to find an increase in the expression of liver genes associated with nitrogen disposal (Fig. 4). Glutaminase mRNA was induced ~2.5- and 2.2-fold in the liver of young and old CR mice, respectively (Fig. 6A). The effect of diet was significant (*P* < 0.001), whereas there was no significant effect of age. CPSI

mRNA also was induced ~2-fold in young and old mice by CR (*P* = 0.0004 and *P* = 0.016, respectively; Fig. 6B). The effects of age and diet were significant (*P* = 0.021 and *P* < 0.001). We have previously shown that the induction of CPSI mRNA in CR mice is accompanied by increased CPSI protein and activity (26). There was a slight (18%) decrease in CPSI mRNA with age (*P* = 0.032), consistent with the age-related decrease in GS mRNA and activity.

TAT is a liver-specific enzyme that provides ketogenic and gluconeogenic substrates when glucose is limiting and amino acids are utilized as a major source of energy. TAT mRNA was significantly induced by CR in young and old mice (*P* = 0.0046 and *P* = 0.026, respectively; Fig. 6C). Aging decreased TAT mRNA in the liver by an average of 37% (*P* = 0.001), whereas CR returned it to youthful levels.

Hepatic glycolytic enzymes. PK catalyzes the third irreversible step in glycolysis, the phosphorylation of ADP to ATP with the utilization of PEP as a high-energy phosphate donor. The reaction produces pyruvate (Fig. 1). In young and old CR mice, LPK mRNA was reduced to 42 and 34% of the level present in control mice (Fig. 7A; *P* = 0.0016 and *P* < 0.0001, respectively). Aging increased hepatic LPK mRNA abundance in control mice (*P* = 0.0016). In contrast, the abundance of MPK mRNA was unchanged by diet or age (data not shown). Consistent with its effect on mRNA, CR also decreased PK activity by 60% in the liver of old mice (Fig. 7B; *P* < 0.001). This decrease in

Table 2. *Renal expression of G-6-Pase and PEPCK mRNA in 7- and 28-mo-old mice*

mRNA	Diet				2-Factor ANOVA P Values	
	Control		CR		Diet	Age
	Young	Old	Young	Old		
G-6-Pase	18.20 ± 4.96 ^b	9.94 ± 1.71 ^a	14.19 ± 4.85 ^{ab}	10.65 ± 2.73 ^a	0.30	0.001
PEPCK	12.79 ± 2.73	8.99 ± 1.22	12.57 ± 2.99	10.43 ± 2.65	0.56	0.009

Values are means ± SD expressed in phosphorimager units normalized to S-II transcription factor. *n* = 6 mice. Mice 7 and 28 mo of age were fed control and CR diets as described in MATERIALS AND METHODS. G-6-Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase. Significance of differences between any 2 groups was tested by Tukey's pairwise comparisons. Within a row, values with the same superscript letter are not significantly different (*P* > 0.05). Where no statistical difference exists between values in a row, no superscript letter is used. Analysis of mRNA levels by 2-factor ANOVA showed no interactions between age and diet for any of the genes listed.

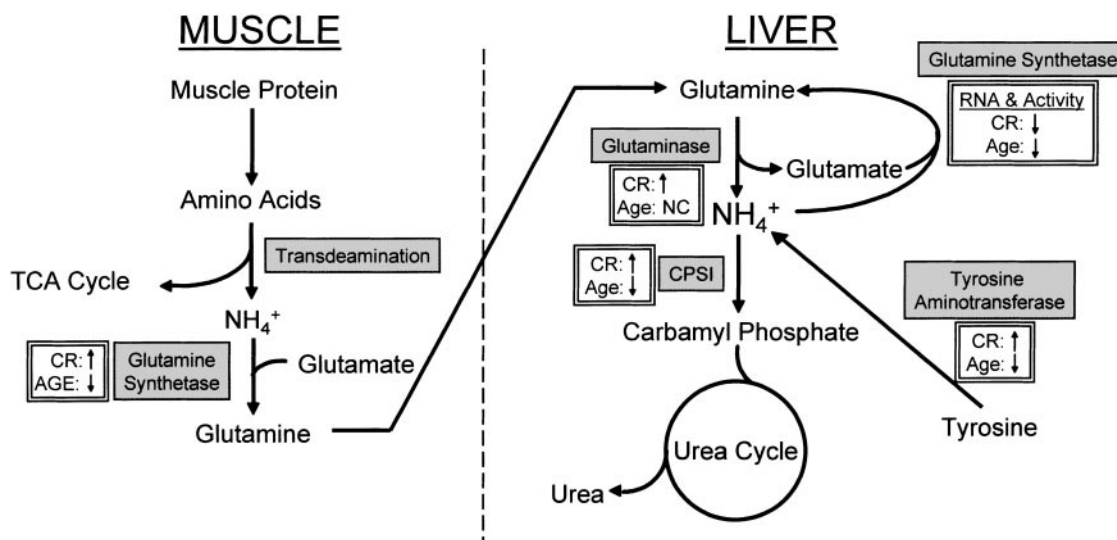


Fig. 4. Summary of effects of age and diet on muscle and liver nitrogen metabolism. Transdeamination of amino acids produces tricarboxylic acid cycle intermediates and ammonia in muscle. Glutamine synthetase synthesizes glutamine from glutamate and ammonia. Glutamine is transported to the liver where glutaminase releases ammonia, regenerating glutamate. CPSI converts this ammonia to carbamyl phosphate, which is converted to urea in the urea cycle. The amino group of excess tyrosine is released by tyrosine aminotransferase (TAT) as ammonia, which is also detoxified beginning with the action of CPSI. Here substrates are not boxed, enzyme names are in shaded boxes, and summaries of experimental results are in double-bordered boxes. When two arrows are given after "CR," they represent degree of change in young and old mice, respectively. An arrow after "Age" is the main effect of age. A down arrow indicates the percent decrease, an up arrow indicates degree of increase. Value given for age is a combination of both dietary groups. NC, no change.

PK activity seems likely to slow the rate of glycolysis in CR mice.

The first committed step in glycolysis is the essentially irreversible phosphorylation of G-6-P by PFK-1 to produce Fru 1,6-P₂ (Fig. 1). PFK-1 mRNA was significantly reduced by an average of 14% in CR mice ($P = 0.02$; Table 1). In these studies, no change was found in the level of GK mRNA with age or diet.

Pyruvate exits glycolysis through oxidative decarboxylation to acetyl-CoA by the enzyme PDH (Fig. 1). CR significantly reduced PDH mRNA by ~14% in both young and old mice ($P = 0.01$, Table 1). Aging had no effect on PDH mRNA abundance ($P = 0.08$; Table 1).

mRNA stability. To investigate the mechanism for the changes in mRNA abundance described in the previous paragraphs, the half-lives of a number of the mRNAs were determined. The levels of specific mRNA were determined at various times after administration of actinomycin D to CR and control mice. The decay

rates of the specific mRNA were used to estimate their half-lives (data not shown). The stability of the different mRNA varied from <2 h, in the case of G-6-Pase, to greater than 24 h for PDH and LPK. However, we found no diet-related differences in the half-lives of any of the mRNAs investigated. These data suggest that the diet-related differences in specific mRNA abundance most likely arise from differences in the transcription rates of the genes. However, the data do not exclude other less common means of regulating mRNA abundance, such as precursor-RNA processing.

DISCUSSION

We have characterized the expression of the key glycolytic, gluconeogenic, and nitrogen-metabolizing enzymes in CR and control mice. The pattern of expression in liver and muscle indicates that CR may reduce the enzymatic capacity of the liver for glycolysis, in-

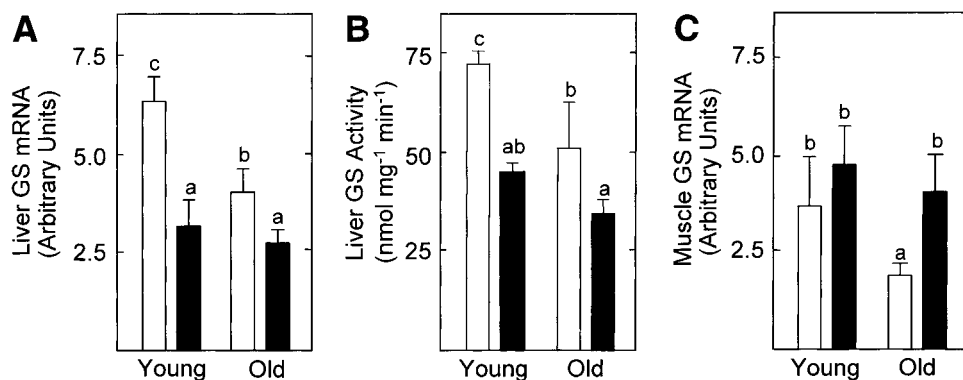
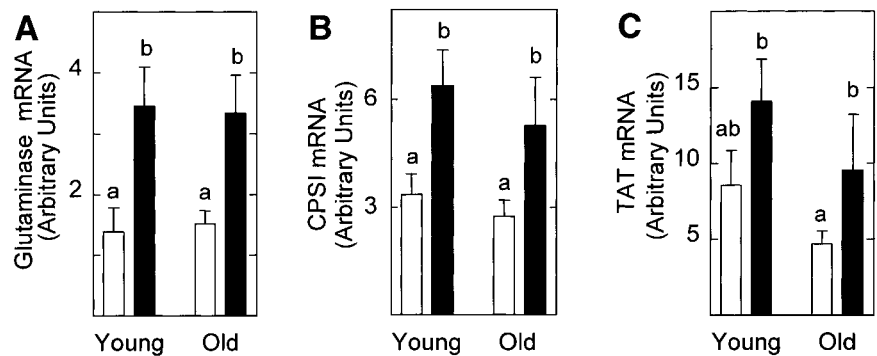


Fig. 5. Effects of age and diet on GS mRNA and activity. Quantitation of liver glutamine synthetase (GS) mRNA (A), liver GS activity (B), and muscle GS mRNA (C) of young and old, control (open bars) and CR (closed bars) mice ($n = 6$ mice for each dietary regimen and age group). Statistical analysis of effects of age and diet is shown as in Fig. 2.

Fig. 6. Effects of age and diet on glutaminase, CPSI, and TAT mRNA levels in liver of young and old mice. Dot-blot quantitation of glutaminase (A), CPSI (B), and TAT (C) mRNA in liver of young and old, control (open bars) and CR (closed bars) mice ($n = 6$ animals from each dietary regimen and age group). Statistical analysis of effects of age and diet is shown as in Fig. 2.



crease the enzymatic capacity of the liver for gluconeogenesis, and increase the enzymatic capacity of liver and muscle for the disposal of nitrogen derived from protein utilization for energy generation. Together, these data support the idea that CR enhances protein turnover at all ages and resists the age-related decline in peripheral tissue protein turnover. Stimulation and continuation of protein renewal in old animals have been proposed as one of the key mechanisms for the anti-aging effects of CR (23, 27).

Enzymes of hepatic gluconeogenesis. After 24 h without food, dietary sources of carbon for maintaining blood glucose levels have been long exhausted. The major source at this time is extrahepatic protein degradation. The only other carbon sources are minor. They are lactate, produced in the muscle by anaerobic metabolism, and glycerol, produced from the degradation of triacylglycerols in adipose tissue.

The doubling of PEPCK mRNA and activity in the liver of CR mice suggests that CR increases the enzymatic capacity for performing the first committed step in gluconeogenesis, the conversion of oxaloacetate to PEP (Figs. 1 and 2). Because our studies are conducted during the postabsorptive phase, oxaloacetate must be chiefly derived from protein turnover in peripheral tissues. There are no known allosteric modifiers of the activity of any PEPCK isoform (4). Therefore, our results are consistent with the idea that CR increases

the utilization of amino acids from peripheral tissues for liver gluconeogenesis.

The decrease in liver PEPCK mRNA with age is similar to that reported in hepatocytes isolated from aging rats (33). The results described here extend these data by showing that the age-related negative regulation extends to muscle PEPCK mRNA, and that CR attenuates this age-related decrease (Fig. 2).

Liver glycogen. Liver glycogen was similarly depleted in control and CR mice after 24 h of food deprivation. Thus differential glycogen depletion is unlikely to account for the differences in the enzymatic capacity for gluconeogenesis found in CR and control mice.

Hepatic glycolysis. The 60% decrease in PK mRNA and activity in CR mice probably decreases the rate of hepatic glycolysis, because PK controls the exit of carbon from the pathway. The statistically significant decrease in liver PFK-1 mRNA in CR mice also may result in decreased enzymatic capacity for glycolysis. Although GK mRNA was not different in CR and control mice after 24 h of fasting, in other studies we have found that CR decreased the induction of GK mRNA by feeding by ~50% (J. M. Dhahbi, P. L. Mote, J. Wingo, S. Cao, B. C. Rowley, R. L. Walford, and S. R. Spindler, unpublished observations). Thus the mRNA and/or activity of all of the key enzymes of glycolysis were reduced in CR mice. Although other studies will be required to determine whether the flux of intermediates through the glycolytic pathway is affected by CR, it seems likely that CR does decrease the enzymatic capacity of the liver for glycolysis.

We found a slight but significant decrease in PDH mRNA in the liver of CR mice. Further work will be required to determine whether this change in mRNA results in a decrease in enzyme activity. However, it suggests the possibility that the enzymatic capacity for directing carbon from glycolysis and gluconeogenesis to acetyl-CoA production also may be diminished in CR mice (Table 1).

Whole body metabolic rate may not be affected by long-term CR (11). However, the techniques used in such studies would discern only substantial changes in energy utilization by major organs. Weindruch and Sohal (30) have suggested that metabolic rates may differ organ-specifically in CR and control animals (30). Our results could be interpreted as support for this idea. We found decreases in the mRNA and/or activity

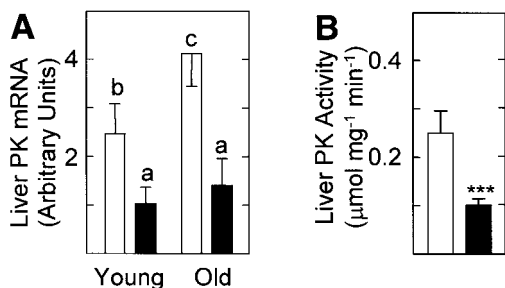


Fig. 7. Effects of age and diet on pyruvate kinase (PK) mRNA and activity in liver and muscle of mice. A: liver PK mRNA levels were quantified in liver from young and old, control (open bars) and CR (closed bars) mice ($n = 6$ animals from each dietary regimen for each age group). Statistical analysis of effects of age and diet are shown as in Fig. 2. B: PK activity in liver from old control and CR mice ($n = 4$ mice in each dietary group). Student's unpaired t -test was used to analyze effect of CR on hepatic PK in this group of mice. Results are very highly significant (***) $P < 0.001$.

of key enzymes for glycolysis and acetyl-CoA formation in the liver, but not in muscle or kidney. Furthermore, MPK activity is nearly 20 times higher than the same activity in liver on a per weight basis (data not shown). It is unlikely that even substantial changes in liver PK activity would be detected in whole body measurements. Thus the possibility for organ-specific changes in energy metabolism should be investigated further.

Nitrogen metabolism. Probably because of its extreme toxicity, the ammonia released by muscle protein catabolism is rapidly transferred to glutamate by GS, producing glutamine (Fig. 4). Glutamine serves as a carrier of nitrogen and carbon between tissues. The carbon is utilized by the liver for gluconeogenesis and the nitrogen for ureagenesis. Hepatic catabolism of glutamine is initiated in periportal hepatocytes by glutaminase (see Fig. 4 and Ref. 12). Ammonia production by glutaminase is coupled to urea synthesis by CPSI.

CR appears to enhance the enzymatic capacity of the liver to dispose of nitrogen derived from amino acid catabolism in extrahepatic tissues. Where the levels of glutaminase mRNA and activity have been reported, changes in the mRNA are always accompanied by equivalent changes in activity (29, 34). Therefore, the CR-related increase in hepatic glutaminase mRNA likely leads to a congruent increase in glutaminase activity. We have reported previously that CR increases hepatic CPSI mRNA and activity to approximately the same extent (26). Therefore, the induction of CPSI mRNA reported here in young and old CR mice is very likely accompanied by induction of CPSI activity (Fig. 6B).

In hepatocytes, nitrogen originating from muscle protein catabolism can be returned to the glutamine pool by the action of GS (Fig. 4). Reduction of GS mRNA and activity in the liver of CR mice should reduce the enzymatic capacity for return of this ammonia and carbon to the glutamine pool. Reduction of hepatic GS activity would favor disposal of nitrogen derived from extrahepatic protein catabolism as urea, and free glutamate for use in gluconeogenesis or protein synthesis.

The induction of GS mRNA in the muscle of old mice is consistent with our other data suggesting that CR increases the enzymatic capacity to metabolize the products of protein catabolism for glucose production. The age-related decrease in muscle GS mRNA in control mice also is consistent with our other data suggesting that there is a decrease with age in the enzymes required to catabolize extrahepatic protein for energy.

In conclusion, the data presented here show that aging was accompanied by a decrease in the expression of genes required for the metabolism of byproducts of amino acid catabolism for energy production in peripheral tissues (decreased muscle GS mRNA and liver CPSI and TAT mRNA), and for gluconeogenesis in the liver (decreased PEPCK and G-6-Pase mRNA). CR generally had the opposite effect of age. CR enhanced the expression of genes required for muscle and liver nitrogen disposal (increased muscle glutamine synthetase mRNA, increased liver glutaminase, CPSI and

TAT mRNA), and decreased the expression of a liver gene essential for mobilization of carbon and nitrogen by the liver for export to extrahepatic tissues (liver GS mRNA and activity). CR also decreased the expression of genes required for glycolysis (reduced PFK-1, PK, and GK mRNA, and PK activity) and for the exit of carbon from glycolysis for the biosynthesis of acetyl-CoA (PDH mRNA). The CR-related changes in the activity and/or mRNA of these enzymes suggest that CR enhances protein turnover in mice of all ages, resisting the well-documented decline in peripheral tissue protein turnover with age. Such an effect on protein renewal may be one of the mechanisms by which CR extends life span.

Address for correspondence and reprint requests: S. R. Spindler, Dept. of Biochemistry, University of California, Riverside, CA 92521 (E-Mail: spindler@ucr.ac1.ucr.edu).

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