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Protein turnover, energy metabolism, aging, and caloric restriction

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Contents

Abbreviations

*CR, caloric restriction; CPSI, carbamylphosphate synthase-1; G6Pase, glucose 6-phosphatase; GS, glutamine synthetase; IGFI, insulin-like growth factor 1; LT-CR, long-term CR; PEPCK, phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase; PFK-1, phosphofructokinase; PK, pyruvate kinase; ST-CR, short-term CR; STZ, streptozotocin; SID, streptozotocin-induced diabetes; TAT, tyrosine aminotransferase; TCA, tricarboxylic acid.

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1. Introduction

Dietary calorie restriction (CR) delays most age-related physiological changes and extends maximum and average life spans in a phylogenetically diverse group of organisms, including homeothermic vertebrates (Spindler, 2003). It is a highly effective means of reducing cancer incidence and increasing the mean age of onset of age-related diseases (Spindler, 2003). Although many of the physiological consequences of CR were described 65 years ago (McCay et al., 1935), there is no consensus regarding its mode of action. However, the metabolic and hormonal changes induced by CR in mammals have been implicated in its age-retarding effects (Weindruch and Walford, 1988).

Changes in the activity of specific genes can control the rate of aging and the rate of development of age-related diseases in invertebrates and mammals (Brown-Borg et al., 1996; Guarente and Kenyon, 2000). In nematodes, life span is regulated by an insulin/insulin-like growth factor receptor homolog, DAF-2. Nematodes with mutations in this signal transduction pathway remain youthful longer, and live more than twice as long as nonmutants. In *Drosophila melanogaster*, a loss of function mutation in the insulin-like receptor homolog gene yields dwarf female flies with up to an 85% extension in adult longevity and dwarf male flies with reduced late age-specific mortality (Tatar et al., 2001).

Similarities between the regulation of aging in invertebrates and mammals suggest that insulin and insulin-like growth factor 1 (IGFI) may have a role in mammalian aging. DAF-2 acts on DAF-16, an HNF-3/forkhead transcription factor family member, to alter energy metabolism and development (Kimura et al., 1997). In mammals, insulin also might mediate its actions on genes such as phosphoenol-pyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT), and IGFI-binding protein-1 through insulin responsive sequences bound by transcription factor complexes containing HNF-3 and other forkhead transcription factors (O'Brien and Granner, 1996; Ghosh et al., 2001). In mice, a family of single-gene mutations which interfere with growth hormone/IGFI signaling and with energy metabolism has been shown to increase mean and maximal life spans by 40–70% (Brown-Borg et al., 1996; Coschigano et al., 2000; Flurkey et al., 2001).

Altered characteristics of fuel use in CR animals have been proposed as a mechanism underlying the anti-aging action of CR (Masoro, 1995). Chronic hyperglycemia is associated with long-term neurological complications, microvascular disorders, basement membrane thickening, and impaired cellular immunity (Rossetti et al., 1990). Hyperinsulinemia is associated with coronary heart disease, hypertension, and atherosclerosis (Stout, 1990). All of the pathologies associated with elevated glucose are reduced or mitigated entirely by CR. In rodents, primates, and humans, CR reduces fasting and average 24-h blood glucose and insulin concentrations, as well as maximum glucose and insulin concentrations during oral glucose tolerance tests (Walford et al., 1992; Harris et al., 1994; Lane et al., 1995).

Whether alterations in glucose utilization and insulin action have a role in determining the rate of aging itself is unknown. To investigate the hypothesis that they have a role, we determined the effects of aging and CR on global patterns

of gene expression using high-density microarrays and conventional molecular biological and biochemical techniques. We found that CR reduces the expression of key enzymes of hepatic glycolysis and increases the expression of key enzymes responsible for gluconeogenesis and the disposal of nitrogen derived from muscle protein catabolism for energy production (Dhahbi et al., 1999; Cao et al., 2001; Spindler, 2001). The studies also showed that CR reverses many of the age-related changes in the mRNA and/or activity of these key metabolic enzymes (Dhahbi and Spindler, 2003). Fasting-refeeding kinetic studies in mice indicate that CR maintains higher rates of gluconeogenesis and protein catabolism, even in the hours after feeding. These data are consistsent with the idea that CR continuously promotes the turnover and replacement of extrahepatic protein into old age (Lewis et al., 1985; el Haj et al., 1986; Merry et al., 1987; Goldspink et al., 1987; Merry and Holehan, 1991; Dhahbi et al., 2001). It appears that in CR animals, protein synthesis immediately following feeding is sufficient to replenish total body protein.

2. Microarrays

Although there have been many studies of the relationship between aging, CR, and hepatic gene expression, there are serious shortcomings to this literature (Dhahbi and Spindler, 2003). There are numerous cross-sectional studies of gene expression in animals of various ages which are interpreted as showing that the major effect of CR is to *prevent* age-related changes in gene expression (Ward and Richardson, 1991). This interpretation has become pervasive in the literature, despite the cross-sectional nature of the studies. Funding and publication bias has reinforced this notion, producing a literature replete with reports of age-related changes in gene expression which appear to be *prevented* by CR.

Genome-wide DNA microarrays are capable of quantifying the expression of all known genes in a single experiment. A significant strength of this approach is the absence of hypothesis-based bias in the choice of genes which are studied. Instead, a comprehensive profile of the relationship between a physiological state and gene expression is generated. Application of this technology has revealed the gene expression signatures underlying the physiological effects of aging, CR, and the dwarf mutations (Golub et al., 1999; Kaminski et al., 2000; Lee et al., 2000; Cao et al., 2001; Kayo et al., 2001; Welsh et al., 2001). In this way, microarrays are providing new insights into aging, the development of age-related diseases, and the ameliorative actions of CR.

Our studies using this technology suggest that rather than simply preventing age-related changes in gene expression, CR instead acts rapidly to establish a new profile of gene expression which may better resist aging (Cao et al., 2001; Dhahbi et al., 2003). Overall, just a few weeks of short-term CR (ST-CR) reproduced nearly 70% of the effects of long-term CR (LT-CR) on genes that changed expression with age (Cao et al., 2001). More recently we have found that essentially all the gene-expression effects of life-long CR can be reversed by just 8 weeks of control feeding (Dhahbi et al., 2003). Thus, CR rapidly induces fully reversible changes in gene expression. Many of these changes are associated with metabolic adjustments to CR.

Approximately, one-third of the CR-specific effects on gene expression are in genes related to energy metabolism and biosynthesis.

3. Aging and energy metabolism

Energy metabolism in the liver is altered by aging. For example, at least two studies have shown decreased mitochondrial respiratory rates in the liver with age (Yen et al., 1989; Muller-Hocker et al., 1997). But, perhaps the major effects of age are on homeostatic glucose regulation. The liver plays a critical role in maintaining glucose homeostasis. This homeostasis is controlled by hormones such as insulin, glucagon, growth hormone (GH), IGFI, and glucocorticoids. High levels of glucose and insulin are implicated in many age-associated pathologies (Rossetti et al., 1990). Likewise, loss of homeostatic glucose regulation is a hallmark of mammalian aging (Halter, 1995). CR reduces blood glucose and insulin concentrations in rodents, primates, and humans (Walford et al., 1992; Harris et al., 1994; Lane et al., 1995). Disorders associated with elevated glucose are reduced or mitigated entirely by CR.

In general terms, our studies of the effects of aging on key hepatic and muscle enzymes of glucose homeostasis indicate that aging is accompanied by a decline in the enzymatic capacity for the turnover and utilization of peripheral protein for the production of metabolic energy (Figs. 1 and 2). We found an age-related decrease in the expression of PEPCK and glucose-6-phosphatase (G6Pase) mRNA in the liver and kidney of mice (Fig. 1) (Dhahbi et al., 1999; Spindler, 2001). An age-related decrease in PEPCK mRNA has been reported by others in isolated rat hepatocytes (Wimonwatwatee et al., 1994). We also found an age-related decrease in PEPCK mRNA in the muscle of mice (Dhahbi et al., 1999).

PEPCK catalyzes the committing step in gluconeogenesis, the conversion of oxaloacetate to phosphoenolpyruvate (Fig. 1). Once carbon is converted to phosphoenolpyruvate, it will be converted to glucose in the liver. PEPCK controls the flow of carbon for hepatic glucose production. This carbon is derived from amino acid intermediates (principally glutamine and alanine) generated by the turnover of protein in the periphery for energy generation. There are no known allosteric modifiers of the activity of any PEPCK isoform (Hanson and Reshef, 1997). This makes PEPCK mRNA and activity levels excellent indicators of the enzymatic capacity of the liver for gluconeogenesis. Thus, aging reduces the gluconeogenic capacity of the liver (Fig. 1).

Liver gluconeogenesis derives its substrates mainly from protein turnover in the muscle and other organs, suggesting that aging is accompanied by a decrease in the turnover of whole-body protein (Goodman et al., 1980). During the postabsorptive state, muscle and other tissues utilize amino acids derived from protein turnover to generate energy via the tricarboxylic acid (TCA) cycle. This amino acid catabolism is initiated in the muscle by two enzymatic steps, collectively called the transdeamination reaction (Fig. 2). Transdeamination leads to the liberation of the amino nitrogens as ammonia. Because of its extreme toxicity, this ammonia is transferred immediately to glutamate by glutamine synthetase (GS), producing glutamine. Glutamine is the major shuttle for nitrogen and carbon

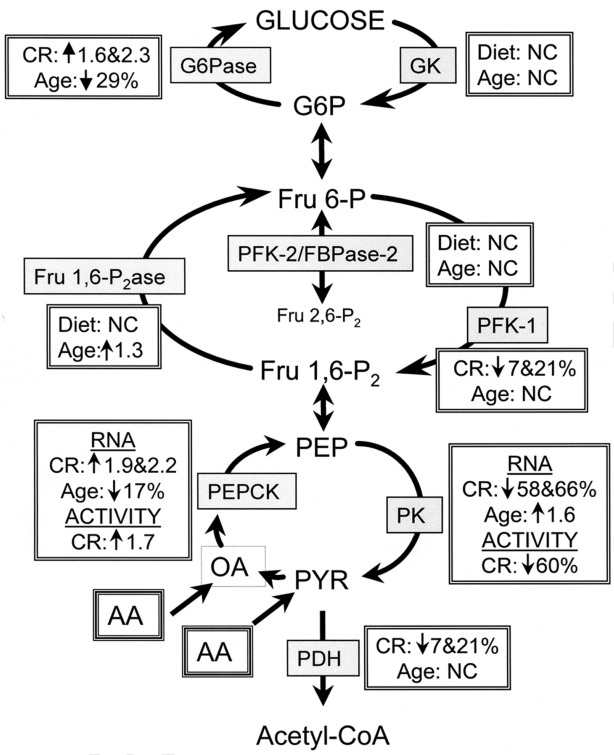

Fig. 1. Summary of the effects of age and CR on the glycolytic and gluconeogenic pathways of the liver. Glycolytic metabolism involves three irreversible, regulated steps. Glucokinase (GK) initiates glucose metabolism by phosphorylation of C6 yielding glucose-6-phosphate (G6P). The committed step in glycolysis, and the second irreversible and regulated step, is the phosphorylation of Fru 6-P by phosphofructokinase (PFK-1) to produce fructose-1,6-bisphosphate (Fru 1,6-P_2). 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, which is a bridge between glycolysis and the tricarboxylic acid cycle. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the first committed step in gluconeogenesis. The main noncarbohydrate precursors for gluconeogenesis are amino acids from the diet and 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-P_2ase) catalyzes the formation of fructose-6-phosphate (Fru 6-P) from fructose-1,6-bisphosphate (Fru 1,6-P_2). Finally, in the third essentially irreversible reaction of gluconeogenesis, glucose is formed by the hydrolysis of G6P in a reaction catalyzed by glucose-6-phosphatase (G6Pase). Substrates are not boxed, enzyme names are in shaded boxes, summaries of experimental results are in double-bordered boxes, and amino acids are indicated by "AA" in triple-bordered boxes. When two values are given following "CR," they represent the fold change in the young and old mice, respectively. The value after "Age" is the main effect of age. A down arrow indicates the percent decrease, an up arrow indicates the fold increase. The value given for age is a combination of both dietary groups. NC is no change.

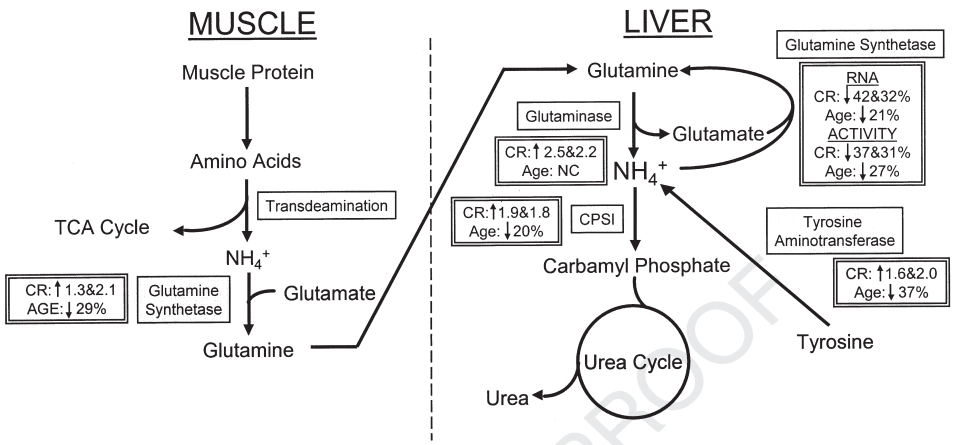

Fig. 2. Summary of the effects of age and diet on muscle and liver nitrogen metabolism. In muscle and other extrahepatic tissues, the degradation of proteins to amino acids is utilized for generating metabolic energy. Transdeamination of amino acids produces TCA cycle intermediates and ammonia. Glutamine synthetase synthesizes glutamine from glutamate and ammonia. Glutamine is transported to the liver where glutaminase releases the 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 TAT as ammonia, which is also detoxified beginning with the action of CPSI. In the figure, substrates are not boxed, enzyme names are in shaded boxes, and summaries of experimental results are in double-bordered boxes. When two values are given following "CR," they represent the fold change in the young and old mice, respectively. The value after "Age" is the main effect of age. A down arrow indicates the percent decrease, an up arrow indicates the fold increase. The value given for age is a combination of both dietary groups. NC is no change.

between tissues in most mammals. It is used by the liver for both gluconeogenesis and ureagenesis. Aging decreases the expression of muscle GS (Dhahbi et al., 1999). This suggests that with age there is a general decline in the enzymatic capacity of the muscle for turnover of proteins.

The differential effects on GS described above should lead to a transfer of carbon and nitrogen in the form of glutamine from the periphery to the liver, where it would increase the hepatic pool of glutamine. Although these studies focused on the liver and muscle, it is very likely that protein degradation declines in this way in all or most tissues of the body. Lewis et al. found a progressive decrease in the rates of whole-body protein synthesis and protein breakdown during aging (Lewis et al., 1985).

These effects are consistent with the decrease in expression of hepatic carbamylphosphate synthase-1 (CPSI), GS, and TAT in the liver of aging mice (Dhahbi et al., 1999, 2001; Spindler, 2001) (Fig. 2). Glutamine produced in the muscle is metabolized in the liver by glutaminase into glutamate and ammonia. The ammonia derived from this reaction can be returned to the glutamine pool by liver GS (Fig. 2). An age-related decrease in GS activity would channel glutamine into gluconeogenesis. The nitrogen from glutamine is channeled by CPSI into the urea cycle for detoxification and disposal. These effects are likely responsible for a part of

the decrease in muscle protein turnover known to accompany aging (Van Remmen et al., 1995).

4. CR and nitrogen metabolism

Our genome-wide microarray studies found that CR modifies the expression of a significant number of key metabolic enzyme genes. ST-CR increases the expression of glutamate oxaloacetate transaminase 1 and decreases the expression of pyruvate dehydrogenase E1α subunit (Cao et al., 2001). CR also induces the expression of three urea-cycle enzymes, arginase 1, argininosuccinate lyase, and argininosuccinate synthetase 1 (Dhahbi et al., 2003). CR further leads to transcriptional induction of CPSI, the enzyme which gates the flow of nitrogen to the urea cycle (Tillman et al., 1996). CPSI enzyme activity is induced five-fold in the liver and two-fold in the small intestine by CR (Tillman et al., 1996). Nitrogen derived from amino acid catabolism in the organs is disposed of in the liver via the urea cycle. Together, these results indicate that CR increases the enzymatic capacity of the liver for the disposal of nitrogen derived from the other organs in the form of glutamine. This glutamine is derived from the catabolism of protein in the organs for energy (Fig. 2).

Consistent with the results described above, CR decreases GS activity and mRNA in the liver (Fig. 2), even immediately after feeding (Dhahbi et al., 2001). CR also leads to higher levels of muscle GS expression (Dhahbi et al., 1999). Together, these results suggest that CR produces a sustained enhancement in the enzymatic capacity for transferring nitrogen and carbon from other tissues to the liver for disposal and gluconeogenesis, respectively.

CR led to a 2.5-fold increase in glutaminase mRNA expression in the liver (Fig. 2) (Dhahbi et al., 1999). Glutaminase mRNA levels closely reflect the levels of glutaminase activity in the liver (Watford et al., 1994; Zhan et al., 1994). Enhanced glutaminase activity should increase the hepatic catabolism of glutamine, producing glutamate and ammonia. Ammonia production by glutaminase is closely coupled to the initiation of urea synthesis by CPSI. As discussed above, CPSI mRNA in young and old mice subjected to CR was 2–5 times the level in control mice (Tillman et al., 1996; Dhahbi et al., 1999, 2001). CPSI responds very rapidly to reduced caloric intake (Tillman et al., 1996). CR leads to coordinate induction of CPSI transcription, mRNA, protein, and activity. The resulting glutamate accumulation in the liver would fuel CR-enhanced gluconeogenesis.

Our microarray studies found that CR increases the expression of cathepsin L, phenylalanine hydroxylase, homogentisate 1,2-dioxygenase, ornithine aminotransferase and histidine ammonia lyase, which are involved in amino acid degradation to provide substrates for gluconeogenesis (Cao et al., 2001). These data support the idea that CR leads to enhanced carbon flux from amino acid degradation in the peripheral tissues to the liver. This amino acid degradation extends to tyrosine, an amino acid that requires a liver-specific enzyme, TAT, for catabolism (Dhahbi et al., 1999). When glucose is limiting, TAT provides ketogenic and gluconeogenic substrates to the liver. Aging decreases TAT mRNA in the liver by an average of 37% (Fig. 2). TAT mRNA in CR mice is approximately double the level in control

316 mice. These results are consistent with the increase in TAT activity in CR rats
317 (Feuers et al., 1989).
318 The changes described above suggest that CR increases the shuttling of nitrogen
319 and carbon from the muscle and organs to the liver. This idea is consistent with the
320 results of Lewis et al., who measured changes in whole-body growth, nucleic acids,
321 and protein turnover with age and CR (Lewis et al., 1985). They found that CR
322 retards the decline in protein turnover with age in rats, and enhances the turnover of
323 whole-body protein during most of the life span. Similarly, Goto et al. found that
324 2 months of CR in old mice significantly reduces the heat liability of proteins in
325 the liver, kidney, and brain, and reverses the age-associated increase in the half-life
326 of these proteins (Goto et al., 2002). ST-CR also reduces carbonylated proteins
327 in liver mitochondria in old rats. These results suggest that CR rapidly reduces
328 the dwell time of the proteins by promoting protein turnover. Consistent with this
329 idea, proteasome activity increases rapidly in the liver of old ST-CR rats (Goto
330 et al., 2002).
331
332 **5. Regulation of nitrogen metabolism**
333
334 The physiological stimuli responsible for the age- and diet-related changes in GS
335 expression have not been investigated. However, during fasting, glucocorticoids
336 mobilize amino acids from muscle protein and increase the rate of glutamine
337 production (Goldberg et al., 1980). CR is associated with daily periods of mild
338 hyperadrenocorticism in rats, and highly elevated midmorning corticosterone levels
339 at all ages in mice (Sabatino et al., 1991; Harris et al., 1994). Glucocorticoids are a
340 robust inducers of muscle GS mRNA (Max et al., 1988). In liver, GS expression is
341 repressed by glucocorticoids (Abcouwer et al., 1995). Thus, it is possible that
342 increased corticosterone levels are responsible for the changes in muscle and liver GS
343 mRNA. Similarly, the decline in circulating corticosterone with age may explain
344 both the age-related fall in muscle GS mRNA and the coordinate increase in liver GS
345 mRNA (Fig. 2).
346 Age- and diet-related changes in corticosterone also could explain the changes
347 found in the expression of other genes. CPSI expression is induced by gluco-
348 corticoids, and the age- and diet-induced alterations in its expression are consistent
349 with the associated changes in serum glucocorticoid levels (Nebes and Morris, 1988).
350 Additionally, GS and CPSI are regulated by GH, glucagon, and insulin (Nebes and
351 Morris, 1988; Palekar et al., 1997). Since CR and aging affect GH and insulin levels
352 and signaling, these hormones or their second messengers also are potential
353 physiological regulators of the genes. We found that GH mRNA is negatively
354 regulated by aging in mice (Crew et al., 1987), and GH levels are well known to
355 decrease with age in mammals and humans. Our recent microarray studies found
356 that the hepatic expression of IGFI-binding protein-1 decreases with age (Dhahbi
357 et al., 2003). This protein plays an important role in the negative regulation of the
358 IGFI system (Frystyk et al., 1999). CR represses expression of GH receptor in the
359 liver of both young and old mice, and induces overexpression of IGFI-binding
360 protein-1 mRNA, which inhibits IGFI signaling.

6. CR and gluconeogenesis

Our microarray studies also showed that CR induces expression of PEPCK and G6Pase, the key gating enzymes of gluconeogenesis (Fig. 1) (Dhahbi et al., 2003). These results confirmed our conventional studies showing that CR induces the fasting levels of G6Pase and PEPCK mRNA, and PEPCK activity (Dhahbi et al., 1999). Hepatic PEPCK mRNA is more abundant in both young and old CR mice than in age-matched control mice. As discussed above, aging decreases the mRNA for PEPCK and G6Pase. In addition, when CR and control mice are fasted overnight, PEPCK mRNA and activity decrease within 1.5 h of feeding in both control and CR mice (Dhahbi et al., 2001). However, its mRNA abundance and activity increased rapidly thereafter, especially in CR mice. By 5 h after feeding, PEPCK activity in CR mice was approximately twice that of controls. Similarly, G6Pase mRNA abundance is higher in CR mice for the 5 h following feeding. G6Pase catalyzes the terminal step in hepatic glucose production, the hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate (Fig. 1). This step leads to the release of glucose from the liver into the circulation.

Liver glycogen is depleted in both control and CR mice after 24 h of fasting (Dhahbi et al., 2001). Furthermore, the extent of this depletion is similar in both groups of mice. In addition, the rate of resynthesis is the same in CR and control mice (Fig. 3) (Dhahbi et al., 2001). These results indicate that the CR-related induction of gluconeogenesis, as evidenced by stimulation of PEPCK activity, is not simply a response to greater glycogen depletion or higher glycogen levels in CR mice. Instead, it probably reflects a metabolic shift to higher rates of protein catabolism to supply substrates for maintaining blood glucose levels.

Together, these results suggest that aging decreases and CR increases the enzymatic capacity for gluconeogenesis and the disposal of the byproducts of extrahepatic protein catabolism for energy production. Furthermore, this capacity

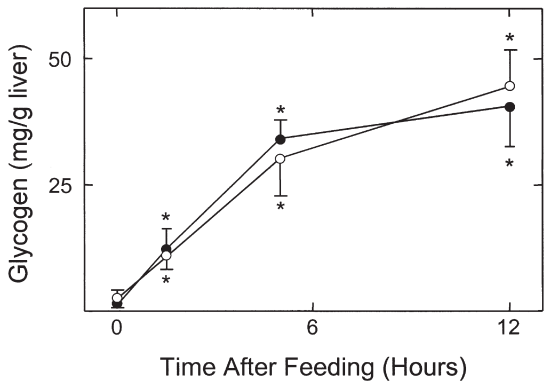

Fig. 3. Glycogen accumulation following food deprivation and feeding in the livers of control (closed circles) and CR (open circles) mice. Hepatic glycogen content was determined after 24 h of fasting (time 0) and at 1.5, 5, and 12 h after feeding[71]. Results are expressed as means ± SD with $n = 5$ mice at each time point. *, Significant difference ($P < 0.001$) relative to time 0 within each dietary group.

returns rapidly after feeding CR mice. However, the return is slower, and less pronounced in control mice, especially old control mice. Thus, higher levels of peripheral tissue turnover persist in CR mice, even after feeding. These mice are at approximate weight equilibrium. Therefore, in CR mice, feeding appears to be accompanied by intensified protein biosynthetic activity followed immediately by peripheral protein turnover. CR mice are approximately four times more insulin sensitive than control mice (Spindler, 2001). Enhanced turnover should reduce the dwell time of proteins in CR mice, and thereby reduce the level and accumulation of damaged protein throughout the body. This effect is consistent with theories of aging, such as the oxidative stress theory, which postulate that the accumulation of damaged proteins contributes to the rate of aging (Stadtman and Berlett, 1998).

7. Regulation of gluconeogenesis

The induction of PEPCK expression in CR rodents may be due to the endocrine changes induced by the dietary regimen. PEPCK mRNA is increased by cAMP, glucocorticoids, and thyroid hormone (Hanson and Reshef, 1997), and decreased by insulin (Hanson and Reshef, 1997). We found that CR reduces blood insulin concentrations by 50% (Dhahbi et al., 2001). Since CR also causes daily periods of mild hyperadrenocorticism, either of these effects could be responsible for increased PEPCK expression. However, insulin is generally regarded as the most potent regulator of its gene activity (Hanson and Reshef, 1997).

Hepatic G6Pase mRNA was ~2-fold higher in the liver of CR mice. Both the human and rat G6Pase mRNA can be induced by glucocorticoids and repressed by insulin and cAMP in hepatoma cells in culture (Lange et al., 1994). Insulin appears to have a dominant role in G6Pase regulation. It suppresses glucocorticoid induction of the gene. Therefore, the most likely explanation for the increased abundance of hepatic G6Pase mRNA in CR mice is reduced insulin levels.

8. Aging, CR, and glycolysis

There are three irreversible, regulated steps in glycolysis. In the first of these, glucokinase initiates glucose metabolism by phosphorylation of glucose at the C6 position, yielding glucose-6-phosphate (Fig. 1). In fasted control and CR mice, we found no difference in the expression of this enzyme (Fig. 1) (Dhahbi et al., 1999). However, 1.5 h after feeding, glucokinase mRNA is induced four-fold in control mice while it is only marginally induced in CR mice (Dhahbi et al., 2001). These results suggest that the glycolytic pathway in CR mice is less responsive postprandially. The results are consistent with the idea that CR reduces the enzymatic capacity of the liver for glycolysis.

The committed step in glycolysis, and the second irreversible and regulated step, is the phosphorylation of fructose-6-phosphate by phosphofructokinase (PFK-1) to produce fructose 1,6-bisphosphate (Fig. 1). PFK-1 mRNA is significantly reduced by

7% in young and 21% in old CR mice (Dhahbi et al., 1999). These results suggest that CR reduces the enzymatic capacity of the liver for glycolysis.

Pyruvate kinase (PK) catalyzes the third irreversible step in glycolysis, the phosphorylation of ADP to ATP utilizing phosphoenolpyruvate as a high-energy phosphate donor. This essentially irreversible reaction controls the outflow of carbon from glucose to pyruvate (Fig. 1). Pyruvate provides carbon for the synthesis of acetyl-CoA. Acetyl-CoA can either go through the TCA cycle, where it generates reducing equivalents for ATP production, or it can provide two-carbon units for fatty acid biosynthesis. CR reduces both PK mRNA and activity by approximately 60% (Fig. 1) (Dhahbi et al., 1999). Similar results have been reported for rats (Feuers et al., 1989). PK activity remains lower in CR mice in the 12 h following feeding (Dhahbi et al., 2001). This change should limit the enzymatic capacity of the liver for glycolysis, and slow the rate of carbon flux through the pathway.

In contrast, to the effects of CR, aging increases liver PK mRNA in control mice. However, muscle PK mRNA levels are not changed by age or diet (Dhahbi et al., 1999). These results suggest that aging is accompanied by an increase in the enzymatic capacity for glycolysis, and increased carbon flux through the pathway to form pyruvate. Because aging decreases mitochondrial respiratory rates in the liver, this increase in the enzymatic capacity for glycolysis likely leads to increased fatty acid biosynthesis (Yen et al., 1989).

Pyruvate exits glycolysis through oxidative decarboxylation by pyruvate dehydrogenase (PDH) to form acetyl-CoA (Fig. 1). CR significantly reduces PDH mRNA by 7% in the young and 21% in the old, while aging has no effect on expression of this enzyme (Fig. 1). PDH activity is reduced two- to three-fold in both fasted and refed CR mice (Dhahbi et al., 2001). Together, these results indicate that the enzymatic capacity for the production of acetyl-CoA from pyruvate is inhibited by CR. This inhibition, combined with reduced GK and PFK-1 expression, should decrease the flux of glucose through glycolysis in the liver of CR mice.

Postprandially, PDH plays a key role in determining whether glucose is used for lipogenesis, the TCA cycle, or glycogen biosynthesis (Holness et al., 1988). The lower levels of PDH activity continuously present in CR mice suggest that the flux of pyruvate into the TCA cycle and fatty acid biosynthesis is limited in these mice. This should allow glycogen to be replenished through gluconeogenesis and the indirect pathway of glycogen biosynthesis (Holness et al., 1988). CR also decreases acetyl-CoA carboxykinase activity (Dhahbi et al., 2001). This change should decrease lipogenesis in CR animals.

9. CR and lipid biosynthesis

In accord with the results discussed above, CR animals have reduced fat mass and decreased levels of serum triglycerides (Stokkan et al., 1991). This reduction in fat biosynthesis is reflected in our microarray studies. CR decreases the expression of acetyl-CoA acetyltransferase 1, fatty acid Coenzyme A ligase, long chain 2, 2,4-dienoyl-CoA reductase 1, liver fatty acid-binding protein-1, hepatic lipase, and

stearoyl-Coenzyme A desaturase 1 (Dhahbi et al., 2003). These changes are consistent with reduced lipid biosynthesis and metabolism. CR also increases the expression of apolipoprotein B-100, a major component of low-density lipoprotein and very low-density lipoprotein (Dhahbi et al., 2003). This increase is consistent with its role in the distribution of hepatic lipid to tissues for use as fuel.

10. CR and protein turnover

Feuers et al. examined the effect of LT-CR on the activities of enzymes supporting glycolysis, gluconeogenesis, and lipid biosynthesis in rats (Feuers et al., 1989, 1990). While the expression of key enzymes was not a focus of their investigation, their conclusions are in close agreement with ours regarding the effects of aging and CR on gluconeogenesis, amino acid metabolism, glycolysis, and lipid metabolism. Lewis et al. found that LT-CR slows whole-body growth and retards the age-related decline in protein turnover (Lewis et al., 1985). They concluded that the CR-related increase in longevity is associated with enhanced protein turnover. Merry et al. studied the influence of aging and CR on protein translation. They found a progressive loss of hepatic translational efficiency with age, and a CR-related enhancement in translational efficiency in rats (Merry et al., 1987). In a related study, Merry and Holehan found that CR reduces the rate of protein synthesis 2.5-fold in young CR rats (Merry and Holehan, 1991). However, by 2 years of age, the rate of protein synthesis in CR rats is significantly greater than in age-matched controls. These studies suggest that CR ameliorates the age-related decline in protein synthesis and turnover. Thus, CR should reduce the dwell time of proteins and the accumulation of damaged proteins with age.

11. Physiological effects of enhanced protein turnover

There is a robust literature documenting elevated levels of oxidized protein, lipid, and nucleic acid with advancing age in rodents (Beckman and Ames, 1998). In general, LT-CR reduces the amount of these oxidized products. Very few of these studies have differentiated the rate of formation from the rate of clearance of these products. Goto et al. found that 2 months of ST-CR in old mice significantly reduces the heat liability of proteins in the liver, kidney, and brain, and reverses an age-associated increase in protein half-life in these tissues (Goto et al., 2002). ST-CR also reduces carbonylated proteins in liver mitochondria of old rats (Goto et al., 2002). Consistent with these results, proteasome activity increases rapidly in the liver of old rats in response to ST-CR (Goto et al., 2002). These results strongly suggest that ST-CR rapidly enhances protein turnover and reduces the dwell time of proteins in the tissues studied.

Sohal and colleagues performed dietary crossover studies in aged calorie-restricted and *ad libitum* fed mice (Dubey et al., 1996; Forster et al., 2000). They found an age-related increase in protein oxidative damage measured as increased carbonyl content and decreased sulfhydryl content in homogenates of brain and heart. This damage was reduced in LT-CR animals. The carbonyl content of whole

brain and the sulfhydryl content of the heart were reduced to LT-CR levels by only 3–6 weeks of ST-CR. Further, a shift from LT-CR to the control diet rapidly increased the level of oxidized protein in brain and heart. Thus, the level of some protein oxidative damage is readily reversible by CR and control feeding.

The results discussed above are consistent with the oxidative stress theory of aging (Harman, 1956; Stadtman and Berlett, 1998). The oxidative stress hypothesis is perhaps the most popular theory of aging at present. A number of investigators have proposed that CR may act by decreasing oxidative damage or enhancing its repair (Sohal and Weindruch, 1996; Yu, 1996). However, until a causal link is established between oxidative damage and aging, the validity of this hypothesis remains unproven.

12. Genome-wide microarray studies of diabetes

We have performed genome-wide microarray studies of streptozotocin (STZ)-induced diabetes (SID) in mice (Dhahbi et al., 2002). STZ selectively destroys pancreatic insulin-producing β-cells, producing a low insulin physiological state characterized by decreased insulin levels, peripheral insulin resistance, and alterations in insulin-dependent signal transduction (Gunnarsson et al., 1974; Yourick and Beuving, 1985; Blondel and Portha, 1989). It is intriguing to compare the gene-expression effects of SID to those of CR, which is also a low insulin state. However, CR is associated with enhanced insulin sensitivity and improved health, while SID leads to enhanced insulin resistance and accelerated development of age-related diseases such as cardiovascular disease.

SID significantly alters the expression of 87 known genes in the liver (Dhahbi et al., 2002). SID increases the expression of genes associated with cytoprotective stress responses, oxidative and reductive xenobiotic metabolism, cell-cycle inhibition, growth arrest, apoptosis induction, and protein degradation. SID also decreases the expression of genes associated with cell proliferation, growth factor signaling, protein synthesis, and xenobiotic metabolism.

It might be anticipated that since both CR and SID are reduced insulin states, their effects on gene expression might be similar. There were limited similarities. Both CR and SID enhance the expression of genes implicated in protein degradation and apoptosis induction (Cao et al., 2001; Dhahbi et al., 2002, 2003). However, other effects are dissimilar. CR generally enhances the expression of cell proliferation, growth factor, growth factor receptor, and protein synthesis-related genes. In contrast, SID inhibits the expression of these categories of genes. CR decreases the expression of genes associated with normal cell-cycle inhibition, growth arrest, and stress responsiveness. In contrast, SID induces the expression of genes important for these processes.

Thus, the gene-expression changes induced by CR are consistent with both enhanced protein degradation and apoptosis, and with enhanced protein synthesis, cell proliferation, and growth factor responsiveness. This is consistent with the results described previously in this chapter. CR appears to generally enhance the turnover and replacement of cells and cellular proteins. In contrast, SID produces

changes in gene expression consistent with enhanced apoptosis, protein degradation, and reduced renewal via cell growth or enhanced rates of synthesis. Thus, SID is generally catabolic in its effects on gene expression.

Hepatocytes are mitotically competent, although they have long, mostly intermitotic life spans. They are exposed to genotoxins from the diet and free radicals generated by xenobiotic metabolism and beta-oxidation. These can lead to the accumulation of damage to proteins, lipids, and nucleic acids, perhaps leading to the impairment of physiological functions and enhanced neoplasia. Apoptosis acts to eliminate damaged and preneoplastic cells, which are then replaced by cell proliferation, thus maintaining homeostatic liver function. Thus, there is an important role for CR-enhanced apoptosis and protein turnover in the maintenance of hepatic function.

13. Conclusions

We have characterized the expression of the key glycolytic, gluconeogenic, and nitrogen-metabolizing enzymes in fasted CR and control mice. The pattern of expression in liver, kidney, and muscle indicates that aging is accompanied by a decline in the enzymatic capacity for the turnover and utilization of protein for the production of metabolic energy. Aging also increases the enzymatic capacity of the liver for glycolysis, probably to provide substrates for fatty acid biosynthesis. In contrast to aging, CR reduces the enzymatic capacity for hepatic glycolysis, and increases the enzymatic capacity for hepatic gluconeogenesis and protein utilization for energy by the liver and extrahepatic tissues. Refeeding studies indicate that CR also increases the enzymatic capacity for gluconeogenesis and the disposal of byproducts of protein catabolism in the hours after feeding. These data are consistent with the idea that CR continuously promotes the turnover and replacement of hepatic and extrahepatic protein. CR appears to enhance protein turnover, even in old age. Comparison of the global gene-expression profiles of CR and SID indicates that each enhances the expression of hepatic genes associated with protein degradation and the induction of apoptosis. However, CR appears to offset these effects with enhanced cell and protein renewal. In contrast, SID appears to both enhance protein degradation and reduce cell and protein renewal. Our results are in agreement with the hypothesis that stimulation of protein renewal is one of the key mechanisms for the anti-aging effects of CR (Stadtman, 1992; Van Remmen et al., 1995).

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