Conserved and Tissue-Specific Genic and Physiologic Responses to Caloric Restriction and Altered IGFI Signaling in Mitotic and Postmitotic Tissues

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Abstract

Caloric restriction (CR), the consumption of fewer calories without malnutrition, and reduced insulin and/or IGFI receptor signaling delay many age-related physiological changes and extend the lifespan of many model organisms. Here, we present and review microarray and biochemical studies indicating that the potent anticancer effects of CR and disrupted insulin/IGFI receptor signaling evolved as a byproduct of the role of many mitotic tissues as reservoirs of metabolic energy. We argue that the longevity effects of CR are derived from repeated cycles of apoptosis and autophagic cell death in mitotically competent tissues and protein turnover and cellular repair in postmitotic tissues. We review studies showing that CR initiated late in life can rapidly induce many of the benefits of lifelong CR, including its anticancer effects. We also discuss evidence from liver and heart indicating that many benefits of lifelong CR are recapitulated in mitotic and postmitotic tissues when CR is initiated late in life.

Contents

INTRODUCTION

Caloric restriction (CR), the consumption of fewer calories while avoiding malnutrition, has long been known to delay many age-related physiological changes and extend maximum lifespan (the average age of the longest-lived 10% of a cohort) and average lifespan in a phylogenetically diverse group of model organisms, including some species of nematodes, flies, and rodents (82). Long-term CR (LTCR), usually begun shortly after weaning, is a highly effective means of reducing cancer incidence and increasing the mean age of onset of many age-related changes and diseases, including immunosenescence, diabetes, renal disease, and some neurodegenerative diseases (82). LTCR, short-term CR (STCR), and a family of longevity-enhancing mutations in mice can dramatically delay tumor-associated mortality and increase apoptosis in at least some mitotic tissues, including the liver and lung (25, 62). Many of the physiological effects of CR were described 65 years ago (85, 86), and the anticancer benefits were described almost a century ago (96). A combination of genetic and other approaches in lower eukaryotes and mammals have identified genetic, metabolic, and hormonal changes that may underlie some of the health and longevity effects of CR (63).

Evolutionary theory holds that responses to CR evolved early in metazoans as an adaptation to boom and bust cycles in the food supply (48, 104). Because selection acts on reproductively active members of a population, it is difficult to rationalize the potent anticancer effects of CR with this theory. Cancer rates are low during the reproductive period of most mammals, and few individuals live long enough in the wild to die of cancer (104). Thus, the anticancer effects of CR may have evolved as a secondary consequence of another trait that was subject to selection. We argue below that this trait is the metabolic role of some mitotic tissues as reservoirs of metabolic energy.

Potent mechanisms of tumor suppression have evolved to suppress the development of cancer until after the reproductive lifespan has ended (see, e.g., 115). Unfortunately, these mechanisms also appear to reduce the potential for tissue regeneration later in life (8, 58, 71, 94). In long-lived rodents, which die primarily of neoplasms, the anticancer effects of CR underlie its lifespan benefits (121). Gerontologists often conjecture that CR slows the "underlying rate of aging" because it increases maximum lifespan. However, in mice, CR can rapidly extend maximum and average lifespan by decreasing the rate of tumor growth (22 and S.R. Spindler, unpublished results). Whether this is rightly viewed as decreasing the rate of aging is open to debate. Thus, it is an open question whether

CR: caloric restriction

LTCR: long-term caloric restriction

STCR: short-term caloric restriction

the concept of "underlying rate of aging," which has never been well defined, has any real meaning.

CONSERVED ADAPTATIONS TO CR IN MITOTIC AND POSTMITOTIC TISSUES

Holliday (48) first proposed that CR is an evolutionary adaptation that diverts limited energy resources from breeding to maintenance. In this part of the review, we present a reanalysis of our published Affymetrix microarray data. It suggests that CR produces a common genic response in heart and liver that is related to a redistribution of metabolic energy. In these tissues, CR appears to decrease protein flux through the endoplasmic reticulum (ER) and Golgi, protein import into the mitochondria, glycoprotein degradation, protein and RNA trafficking between the nucleus and cytoplasm, nucleotide and nucleic acid metabolism, and inflammation. These results are consistent with the general reduction in the rates of protein, RNA, lipid, and DNA synthesis that are found in CR animals (e.g., 14, 15, 75, 89). We argue here that it is the role of tissue protein and lipid as reversible sources of metabolic energy that leads to many of the anticancer and longevity-related effects of CR.

Microarray Data Analysis

The data obtained in large-scale microarray studies is often misinterpreted. Gene lists produced using different analytical platforms and statistical tools are sometimes compared to identify similarly changed genes. One such comparison of microarray results obtained with tissues from mice, rats, pigs, monkeys, yeast, and flies found no common genes that were responsive to CR (43). But, this result would be expected, even if similarly changed genes exist. All large-scale microarray studies require sophisticated analytical and statistical tools to remove false positives and maximize the number of real positives identified. As the statistical stringency of the analysis increases to cull false positives from the set of found genes, fewer real positives remain. Further, even when identical data sets are analyzed with different statistical tools, only partially overlapping gene sets are identified (110). For example, we used two different statistical methods on a single data set and only found ~50% overlap between the sets of genes that were identified (28).

A better way to make such comparisons is to use data from a single array platform, globally normalize all probe set results, and apply identical analytical and statistical tests to all the normalized data. We used this approach to reanalyze our Affymetrix data from heart and liver (14, 22, 23, 28, 126). This new analysis identified 32 genes that were similarly responsive to CR in heart and liver (**Table 1**). All but two of these genes were downregulated. The genes fall into a number of functional groups (**Tables 1** and **2**).

Conserved Responses

CR decreases the rate of DNA and protein synthesis and decreases the RNA content of the liver, kidney, heart, and small intestine in rats (32, 38, 75, 88–92). Despite decreased synthetic rates, LTCR animals appear to maintain their organ mass by decreasing the rate of protein degradation (see below). In LTCR animals, the sum of these effects appears to produce higher rates of protein turnover and smaller visceral organ sizes (132). CR begun in older control mice reduces organ sizes and increases the rate of protein turnover (see below).

Chaperones. LTCR downregulated five major cytoplasmic and ER chaperones in heart and liver (**Table 1**). This was found even though LTCR protects cardiomyocytes from apoptotic and necrotic cell death throughout life (28). The three ER chaperone genes downregulated in liver and heart, *Hspa5* (also known as GRP78 or BiP), *Calr* (calreticulin), and *Pdia3* (Grp58), have major roles

ER: endoplasmic reticulum

Table 1 Genes changed by long-term caloric restriction in both liver and heart[a]

Symbol	Name	Heart CR/Con FC[b]	Liver CR/Con FC	Function
Glycoprotein catabolism				
Aga	Aspartylglucose-aminidase	−1.6	−1.4	One of the final steps in lysosomal breakdown of glycoproteins. It cleaves the amide bond between asparagine and the oligosaccharide.
Aspa	Aspartoacylase (aminoacylase) 2	−1.4	−1.3	Nuclear and cytoplasmic membrane enzyme that catabolizes the terminal N-acylpeptides or N-acylated amino acids. It is a scavenger of N-acetylaspartic acid.
Asrgl1	Asparaginase-like 1	−1.3	−1.3	Mainly a mitochondrial enzyme that catalyzes the conversion of L-asparagine to aspartic acid and ammonia.
Dpp3	Dipeptidylpeptidase 3	−1.4	−1.3	Releases an N-terminal dipeptide from a peptide composed of four or more amino acids, including angiotensin and enkephalin. Has a broad specificity.
Fbxo6b	F box–only protein 6b (Frap)	−1.5	−1.4	ER-associated enzyme that targets sugar chains in N-linked glycoproteins for ubiquitination and degradation. Involved in ER quality control and the calnexin-calreticulin cycle. Plays a role in the differentiation and hepatocyte proliferation.
Hexa	Hexosaminidase A (alpha polypeptide)	−1.7	−1.5	α subunit of the lysosomal β-hexosaminidase that catalyzes the degradation of molecules containing terminal N-acetyl hexosamines.
Manba	Mannosidase, beta A, lysosomal	−1.4	−1.3	Catalyzes the penultimate step in lysosomal N-linked oligosaccharide catabolism. Cleaves the single β-linked mannose residue from the nonreducing end of all N-linked glycoprotein oligosaccharides.
Nagk	N-acetyl-glucosamine kinase	−1.5	−1.4	Converts endogenous GlcNAc produced by lysosomal degradation or from nutritional sources into GlcNAc 6-phosphate, which can enter further catabolic or anabolic pathways.
Protein processing/repair				
Mipep	Mitochondrial intermediate peptidase	−1.6	−1.4	Releases an N-terminal octapeptide as the second stage in processing some proteins imported into the mitochondria.
Msrb2	Methionine sulfoxide reductase B2	−1.3	−1.4	Mainly a mitochondrial enzyme that repairs oxidative damage to methionine residues. Implicated as one of the primary defenses against oxidative stress.
Psen2	Presenilin 2	−1.8	−1.4	ER and *cis*-Golgi localized, catalytic subunit of the endoprotease γ-secretase complex that catalyzes the intramembrane cleavage of integral membrane proteins such as the Notch receptors and the β-amyloid precursor protein.
Chaperones				
Calr	Calreticulin	−1.3	−1.4	Major lumenal ER chaperone. Major Ca^{2+}-binding and storage protein. It has a key role in the calreticulin/calnexin quality control cycle. In the nucleus it inhibits retinoic acid, glucocorticoid, and androgen receptor action.

(*Continued*)

Table 1 (*Continued*)

Symbol	Name	Heart CR/Con FC[b]	Liver CR/Con FC	Function
Fkbp3	FK506-binding protein 3; peptidylprolyl isomerase; cyclophilin; (rotamases)	−1.3	−1.3	Nuclear immunophilin involved in protein folding and trafficking. Its inhibition in cancer cells suppresses proliferation, the transformed phenotype, and tumorigenicity.
Fkbp5	FK506-binding protein 5; peptidylprolyl *cis*-trans isomerases (rotamases)	2.0	2.1	Immunophilin involved in protein folding and trafficking. It is part of a heteromultimeric cytoplasmic complex with HSP90, HSP70, and some steroid hormone receptors. Dissociates when glucocorticoid receptor binds its ligand. Induced by progestins and androgens.
Hspa5	GRP78; BiP; heat shock 70kD protein 5	−1.8 to −1.5	−1.6	Major lumenal ER chaperone. When induced can be antiapoptotic by binding some caspases.
Pdia3	GRP58; protein disulfide isomerase family A, member 3	−1.9	−1.4	Major luminal ER protein disulfide isomerase that binds calreticulin and calnexin to modulate folding of newly synthesized glycoproteins. Involved in quality control. Interacts with the DNA-binding domain of the glucocorticoid receptor to mediate its nuclear export.
Triap1	TP53-regulated inhibitor of apoptosis 1	−1.6	−1.3	p53-inducible gene involved in the p53-dependent cell-survival pathway. In response to low levels of DNA damage, it inhibits apoptosis by inhibiting activation of caspase-9.
Transporters				
Slc44a1	Solute carrier family 44, member 1	−1.4	−1.3	Probable choline transporter in the plasma membrane. Choline is a major source of methyl groups that participates in S-adenosylmethionine biosynthesis.
Inflammation				
Comt	Catechol-O-methyltransferase	−1.6	−1.3	Involved in catecholamine degradation and in detoxification. Likely low because serum catecholamine levels are low.
Hpgd	Hydroxyprostaglandin dehydrogenase 15 (NAD)	−1.4	−1.5	Rate-limiting enzyme for PGE2 and PGF2α catabolism. Likely low because PGE2, which is produced by macrophages, is low.
Il13ra1	Interleukin-13 receptor alpha-1 chain precursor	−2.7	−1.4	Plasma membrane receptor capable of transducing a proliferative signal in response to IL13. In eosinophils, IFNG, TNFA, and particularly TGFB enhance its expression. Overexpressed in tumor cells.
Lyzs	Lysozyme (renal amyloidosis)	−2.2	−1.6	A myeloid cell-specific marker induced by macrophage activation.
Protein and nucleic acid nuclear export/import				
Cblb	Casitas B-lineage lymphoma b; Cas-Br-M (murine) ecotropic retroviral transforming sequence b	1.6	1.3	Key role in protein import into the nucleus. E3 ubiquitin-protein ligase accepts ubiquitin from specific E2 ubiquitin-conjugating enzymes and transfers it to substrates, generally promoting their degradation by the proteosome.

(*Continued*)

Table 1 (*Continued*)

Symbol	Name	Heart CR/Con FC[b]	Liver CR/Con FC	Function
Ddx39	DEAD (Asp-Glu-Ala-Asp)-box polypeptide 39	−1.3	−1.4	Involved in pre-mRNA splicing and the export of mature mRNA from the nucleus. Upregulated in proliferating cells. Lower levels in quiescent cells.
G3BP2	Ras-GTPase activating protein SH3 domain-binding protein 2	−1.4	−1.3	Implicated in Ras, NFκB signaling, the ubiquitin proteasome pathway, and RNA processing. Retains IκBα and IκBα/NFκB complexes in the cytoplasm.
Ipo11	Importin 11	−1.4	−1.3	Functions in nuclear protein import as a receptor for nuclear localization signals in cargo substrates. Thought to mediate docking of the importin/substrate complex to the nuclear pore complex.
Ncbp1; Reflbp2	Nuclear cap–binding protein subunit 1, 80 kDa	−1.6	−1.3	Present in a U snRNA export complex with m7G-capped RNA. Subunit of the exon junction complex.
Pttg1ip	Pituitary tumor–transforming 1 interacting protein precursor	−1.3	−1.5	A multifunctional human securin oncogene, with roles in mitosis, cell transformation, DNA repair, gene regulation, and fetal development. Overexpression has been reported in multiple tumor types. Interacts with p53 and blocks its activity by blocking its binding to DNA.
Nucleotide/nucleic acid metabolism				
Dtymk	Thymidylate kinase; dTMP kinase	−1.7	−1.4	Thymidylate kinase activity parallel rates of cell cycle progression and growth.
Nudt1	Nudix (nucleoside diphosphate-linked moiety X)-type motif 1	−1.5	−1.7	Hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, preventing its misincorporation into DNA, which would lead to A:T to C:G transversions.
Nudt2	Nudix (nucleoside diphosphate-linked moiety X)-type motif 2	−1.4	−1.5	Asymmetrically hydrolyzes Ap4A to yield AMP and ATP. Plays a major role in maintaining nucleotide homeostasis. Ap4A and other dinucleotides participate in blood pressure regulation.
Pnkp	Polynucleotide kinase 3′-phosphatase (ATP-dependent poly-deoxyribonucleotide 5′-hydroxyl-kinase) Ap4a (murine)	−1.6	−1.4	Catalyzes the phosphorylation of nuclear DNA at 5′-hydroxyl termini and can dephosphorylate the 3′-phosphate termini. Has an important function in DNA repair following oxidative damage.

[a]Probe set data from our published studies were reanalyzed to identify genes that were similarly responsive to LTCR in both liver and heart (14, 22, 28, 122, 126). The Entrez, GeneCard, Bioinformatic Harvester, NCBI OMIM, and PubMed databases were used to identify functions.
[b]Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; Con, control; CR, caloric restriction; ER, endoplasmic reticulum; FC, fold change; LTCR, long-term caloric restriction.

in the ER stress response, protein folding, glycosylation, the calreticulin/calnexin cycle, and quality control. We previously reported that STCR and/or LTCR downregulate many ER and several cytoplasmic chaperones in the liver, skeletal muscle, kidney, and heart of mice (14, 20–22, 24, 28, 123, 126). A number of ER and cytoplasmic chaperones

Table 2 Comparison of the categories of the genes altered by long-term caloric restriction and short-term caloric restriction in the liver and heart of mice

Liver specific[a]	Conserved in liver and heart	Heart specific
Down[b]	**Down**	**Down**
Glycolysis	Chaperones and stress response	Fibrosis- and tissue-remodeling related
Intracellular signaling	Glycoprotein catabolism	Extracellular matrix, cytoskeletal structure and dynamics
Up	Protein and RNA nuclear import and export	Cell motility
Fatty acid oxidation and lipid catabolism	Inflammation and immune activation	Blood pressure and hemodynamic stress
Gluconeogenesis	**Mixed**	Signal transduction, differentiation, cell division
Proapoptotic	Xenobiotic, oxidant and toxicant metabolism	Protein degradation
Ureagenesis		**Up**
		PPARα signaling (enhanced energy homeostasis)
		cAMP (enhanced cardiac contractility)
		Mixed (but mostly downregulated)
		Proapoptotic

[a]The genes were assigned to functional groups by reference to our previous publications (14, 22, 28, 122, 126) and using the Entrez, GeneCard, Bioinformatic Harvester, NCBI OMIM, and PubMed databases.
[b]Down, decreased by CR; up, increased by CR relative to control; mixed, genes were both up- and downregulated.

are additively downregulated by the combination of LTCR and the Ames dwarf mutation (126). Ames dwarf mice are homozygous for a loss-of-function mutation in the Prop1 gene, leading to reduced serum levels of insulin like growth factor I (IGFI) and other hormones produced by or released in response to signaling from the anterior pituitary (119). Fasting also downregulates and refeeding upregulates cytoplasmic and ER chaperones, possibly in response to changes in blood insulin and glucagon levels (20, 21). Two chaperone genes recently were reported to be downregulated by CR in mouse heart, liver, and hypothalamus (37). These results are consistent with a reduced level of protein synthesis and trafficking in the mitotic and postmitotic tissues of fasted, CR, and Ames dwarf mice.

It is often posited that chaperone overexpression should extend lifespan (e.g., 44, 45). Transient induction of heat shock proteins does extend the lifespan of nematodes (101). However, it is highly unlikely that heat shock proteins [which are undetectable at all times in control and CR mice (unpublished results)] are ever induced in animals that are conventionally maintained in a vivarium. Many of the chaperones that normally are expressed in vivo are downregulated by LTCR, STCR, and starvation, as described above. There is no reason to expect that constitutive overexpression of chaperones should extend mammalian lifespan. Chaperones are pleiotropic in their actions. Some initiate protein degradation as well as protein folding and maturation (113). A number of chaperones bind caspases, negatively regulate apoptosis, and promote carcinogenesis (109, 113, 137). For example, calreticulin has roles in secretory pathway quality control, intracellular Ca^{2+} homeostasis, ER Ca^{2+} storage, early cardiac development, autoimmunity, and cancer (134). In mice, most chaperones are downregulated by LTCR at all times, especially before feeding (20, 21, 24, 97). Feeding induces the expression of some chaperones, matching their abundance to that of their substrates, which are postprandially synthesized proteins (20, 21).

IGFI: insulin-like growth factor I

Intracellular protein and RNA trafficking. Seven glycoprotein-catabolism–related genes were downregulated in heart and liver (**Table 1**). Downregulation of the *Psen2* (presenilin 2) gene is consistent with the general downregulation of protein trafficking in the ER. Presenilin 2 is the catalytic subunit of the γ-secretase complex, the maturase that processes membrane proteins like β-amyloid precursor protein and the Notch receptors. Together, these results suggest that CR reduces the rates of glycoprotein synthesis and catabolism in heart and liver. These changes must be viewed in the context of a general decrease in macromolecular synthesis and turnover, which nonetheless results in enhanced rates of protein turnover for energy production (see below). Pulse-chase studies in hepatocytes isolated from LTCR mice demonstrated that downregulation of ER chaperones paradoxically increases the efficiency of trans-ER-Golgi transport and protein secretion, probably by decreasing protein degradation in the ER (21). Thus, the liver appears to adapt to reduced dietary energy by increasing the efficiency of protein secretion.

Further evidence for reduced synthesis and trafficking of macromolecules in the liver and heart of CR mice comes from the downregulation of six genes involved in protein and RNA import and export from the nucleus, and four genes involved in DNA and RNA synthesis and repair (**Table 1**). For example, the product of the downregulated Ddx39 gene, an RNA helicase, is required for pre-mRNA splicing and nuclear export. It is downregulated in quiescent cells, consistent with other data suggesting there is a decrease in the rate of cell growth and division in CR animals (46, 61). However, despite decreased rates of cell division in CR animals, the liver cells of CR rats appear to respond more promptly to toxic challenge and promitogenic signaling than those of controls (4).

Inflammation. The downregulation of four inflammation-related genes in LTCR liver and heart is consistent with the reduced inflammation often reported for LTCR mice (15; **Table 1**). For example, Lyzs (lysozyme) is an inducible marker of macrophage activation and inflammatory disease (16, 64). IL13R (interleukin 13 receptor) is overexpressed in human tumor cells (116), and IL-13 is a major inducer of fibrosis through induction of transforming growth factor β1 in macrophages (34). Thus, downregulation of these genes is consistent with reduced inflammation and macrophage activation in the heart and liver of CR mice. Other conserved changes reported above may represent changes in macrophage and/or white blood cell–related gene expression.

CR, CANCER, AND MITOTIC TISSUES

We do not know how CR extends the lifespan of any organism, not even that of the intensively studied model organisms such as *C. elegans* and mouse. Genetic studies in *C. elegans* have elegantly defined genes and genetic pathways capable of extending lifespan (63). However, we do not know why nematodes die of old age; therefore, we do not know how these longevity pathways extend lifespan. There is a similar problem with mice. Most laboratory strains of mice die of a few, strain-specific types of tumors, often hepatomas and/or lymphomas (22, 121, 127). However, since the discovery of the potent anticancer effects of LTCR nearly a century ago, there have been almost no mechanistic studies of how CR prevents death from the spontaneous tumors that actually kill the mice. Essentially all CR-related studies of cancer use transplanted or induced tumors (53). Although this work has been illuminating, there is no clear evidence that these models recapitulate the mechanisms at work in the spontaneous tumors that actually limit, and thereby determine, the lifespan of mice.

As discussed above, a decrease in the rate of tumor growth increases the maximum lifespan of mice. The extension of maximum lifespan is normally thought to be indicative of

decelerated aging. However, for the reasons discussed above, screening for longevity therapeutics in mice will likely identify mostly compounds that intervene in tumorigenesis (23, 122). Paradoxically, this may also identify therapeutics with other longevity-enhancing effects. Many of the molecular pathways controlling lifespan in nematodes, which do not normally die of tumors, also reduce the growth rate of a mutationally induced tumor in this organism (106). Thus, the ancient pathways controlling longevity in metazoans are highly pleiotropic in their effects. Most of the interventions known to extend lifespan also alter key signaling systems controlling the expression of relatively large batteries of "effector" genes (7, 76, 126). Thus, the pathways capable of extending lifespan in metazoans appear to have been selected for their ability to directly target the wide array of disease processes that lead to death.

CANCER, CR, AND REDUCED ANTERIOR PITUITARY SIGNALING

Relatively few cancers arise from postmitotic cells (135). Cell division is required to genetically fix oncogenic mutations. As discussed above, selection acts on reproductively active members of a population, and few mice live long enough to die of cancer in the wild (104). Highly effective molecular mechanisms have evolved to suppress the development of cancer until after reproduction ceases. These include mechanisms for high fidelity DNA replication and repair, and tumor suppressor genes such as $p16^{INK4a}$ and ARF. The expression of $p16^{INK4a}$ and ARF increases with age in stem and possibly other cell types, upregulating the retinoblastoma and p53 pathways, respectively (58, 71, 94). In mice, this upregulation may suppress the development of cancer until after the end of the reproductive lifespan (115). Thus, the anticancer effects of CR may have coevolved with another phenotype, which was subject to natural selection. Below we discuss published studies suggesting that CR drives higher rates of protein and lipid turnover in postmitotic and mitotic tissues, and the apoptotic and/or autophagic turnover of cells in mitotically competent tissues. We postulate that this turnover drives many of the beneficial effects of CR on health and lifespan.

A widely accepted model proposes that carcinogenesis involves three steps: initiation, an initial mutation in a tumor suppressor gene; promotion, the accumulation of mutations in cell growth or proliferation-related genes; and progression, a gain in tumor mass through increased rates of cell proliferation and/or reduced rates of apoptosis (53). The relative rates of proliferation and apoptosis during promotion and progression are major determinants of the rates of tumor onset and growth. LTCR reduces carcinogenesis at every stage of this model (53). LTCR increases the age of onset and decreases the rate of growth and number of metastases produced by model tumors, including hepatomas, mammary carcinomas, and prostatic tumors (53, 133). LTCR suppresses the carcinogenic action of several classes of chemicals, inhibits several forms of radiation-induced cancer, and inhibits neoplasia in early-tumor-onset knockout and transgenic mouse models (53). There is compelling evidence that in mitotic tissues, LTCR enhances the rate of apoptosis in preneoplastic, tumor, and normal cells. Preneoplastic and tumor cells appear to be more susceptible to apoptosis than normal cells. For example, the rate of apoptosis, as measured using terminal dUTP nick end labeling (TUNEL) of apoptotic bodies, is three times higher in hepatocytes of CR mice at all ages (55, 56, 98). Increased hepatocyte apoptosis is associated with a significantly lower incidence of spontaneous hepatomas throughout the life of LTCR mice, although the mechanism remains unknown. Even brief periods of CR enhance apoptosis and reduce tumor incidence. For example, one to three months of food restriction can significantly increase the latency and reduce the incidence of spontaneous cancer over the entire lifespan of a mouse (65). Just one week of CR induces

apoptosis of the glutathione S-transferase-II-positive (an immunohistochemical marker of preneoplastic liver cells) hepatocytes of old mice (99). Forty-percent food restriction for three months eliminates 20% to 30% of liver cells through apoptosis and reduces the number and volume of chemically induced preneoplastic foci by 85% (40). CR also enhances apoptosis in mitotically competent cells of other organs, including jejunum, colon, bladder, and dexamethasone-treated lymph node and spleen lymphocytes of MRL/lpr mice (49, 78). We found that CR begun at 19 months of age in C3B6F1 mice (at the beginning of the accelerated mortality phase of their lifespan) decreases tumor-associated mortality by 3.1-fold within eight weeks and extends both mean and maximum lifespan (22, 121). These effects appear to be due to a reduction in the rate of tumor growth (22, 121). Because CR does not appear to reduce the rate of tumor-cell division, it probably increases their rate of apoptosis or autophagic cell death (S.R. Spindler, Y. Higami, & I. Shimokawa, unpublished results).

Tumorigenesis can involve inactivation of tumor suppressor genes, activation of oncogenes, overexpression of growth factors, and inappropriate growth factor signaling. LTCR, STCR, methionine restriction, and a family of mutations in mice dramatically extend lifespan and reduce insulin and IGFI serum levels and postreceptor signaling (6, 7, 25, 62, 68, 80). They also delay tumor-associated mortality and increase apoptosis in mitotic tissues, including the liver (25, 29, 62, 67). The IGFI receptor plays the major role in mitogenesis, transformation, tumorigenicity, and protection from apoptosis in vivo (1, 2, 12, 50–52, 103). For example, neoplastic lesions and the incidence of adenocarcinoma are reduced in lung, and the growth of spontaneous and transplanted tumors is reduced in Ames dwarf mice (54). Serum IGFI levels are reduced by many of the interventions that extend mammalian lifespan in rodents (6, 7, 76, 111). In tumor cells, IGFI acts as an autocrine/paracrine growth factor as well as an inhibitor of apoptosis (111). IGFI receptor is emerging as a critical factor in hepatocarcinogenesis (112). Defects in IGFI receptor expression and/or activation inhibit tumorigenicity, reverse the transformed phenotype, and cause massive apoptosis in vitro and in vivo (13, 111). CR has a well-described anticarcinogenic effect on spontaneous and chemically induced tumors (53). Reduced proliferation and increased apoptosis are thought to be responsible (53). Downregulation of fatty acid synthase and fatty acid biosynthesis by CR and the dwarf mutations also may be a source of its antioncogenic effects (14, 72, 108, 126). Both are required for the survival of many human cancer cell lines. The inhibition of fatty acid synthase rapidly leads to apoptosis in tumor cells (72, 108).

In one genomewide microarray study of liver gene expression, we found that 21% of the genes that changed expression in response to LTCR are associated with apoptosis, cell growth, or cell survival (14). For example, LTCR induced the expression of the BAK1 and VDAC1 genes. The products of these genes interact to release cytochrome c from mitochondria, initiating apoptosis (117). The rapid induction of Vdac1 after four weeks of CR is consistent with the increase in apoptosis and reduction in chemical carcinogenesis also found in fasting rodents (14, 47, 65, 107).

PROTEIN TURNOVER AND GLUCONEOGENESIS

Cellular turnover in metazoans involves degradation of cytoplasmic and nuclear proteins by cellular calpains (118) and the proteosome (100); degradation of mitochondria by mitochondrial proteases (5); autophagic degradation of membranes, mitochondria, ribosomes, ER, and peroxisomes (10, 30, 60); and cellular turnover initiated by apoptotic, necrotic, and autophagic cell death (59). Nutritional stress increases the rate of these processes (35). LTCR (30%) reduces the weight of the heart, liver, kidney, spleen, prostate, and skeletal muscle of rats by 25% to 50% (132).

This reduction appears to be due to both decreased rates of macromolecular synthesis and cell division (32, 38, 75, 88–92) and increased rates of cell death and macromolecular degradation. Mitotically competent tissues such as liver and lung undergo a profound, rapid, and reversible loss of cell number (via necrosis, apoptosis, and/or autophagic cell death), protein, and lipid after the initiation of either CR or fasting (69, 83). For example, fasting for 48 hours reduces liver weight by half and liver proliferative index by 85% while increasing its apoptotic index by 2.5-fold (70). The number of lung alveoli in mice is reduced by 35% after 72 hours of 33% CR (83). Fifteen days of CR reduces alveolar number by 45% (83). Refeeding for 72 hours fully restores alveolar number (83). Thus, CR appears to tip the regulatory balance in some mitotic tissues toward apoptosis and the degradation of cellular carbohydrate, protein, and lipid.

AGING AND ENERGY METABOLISM

Aging produces a decline in the autophagic and apoptotic turnover of cells, organelles, membranes, carbohydrates, and proteins (10, 30). It also decreases the enzymatic capacity for utilizing protein for the production of metabolic energy (120). During the postabsorptive state, when blood insulin and glucose levels begin to wane and glucagon, glucocorticoids, and catecholamines increase, most tissues begin to utilize glycogen and amino acids derived from protein turnover to generate energy via the tricarboxylic acid cycle. This drives the autophagic degradation of proteins, organelles, and membranes. Amino acid catabolism is initiated by two enzymatic steps, collectively called transdeamination (**Figure 1**). The enzymatic capacities for many of these transdeamination reactions are enhanced in the liver of LTCR mice (41). Transdeamination leads to the liberation of the amino nitrogen as ammonia, which is transferred to glutamate by glutamine synthetase (GS) to produce glutamine. Glutamine is the major shuttle for nitrogen and carbon between tissues in most mammals. In the liver, it is used for both gluconeogenesis and ureagenesis. Aging decreases the expression of muscle GS in mice (26), suggesting that the enzymatic capacity for the metabolism of the products of protein turnover decreases with age. In rats, muscle GS levels increase with age, except for 55% of the oldest animals, where levels are reduced (105). These results suggest that the enzymatic adjustments to muscle protein turnover with age may be more complex in rats than in mice.

CPSI: carbamylphosphate synthase-1

Glutamine produced in muscle is metabolized by glutaminase in the liver to glutamate and ammonia (**Figure 1**). Because of its extreme toxicity, the ammonia is channeled into the urea cycle for detoxification and disposal. The decrease in muscle GS found in aged mice is consistent with the decrease in hepatic carbamylphosphate synthase-1 (CPSI), GS, and tyrosine amino transferase with age (14, 25, 26, 120, 125; **Figure 1**).

Glutamate (and other amino acids) undergoes transdeamination in the liver and enters the tricarboxylic acid cycle as oxaloacetate (or pyruvate; **Figure 2**). Once oxaloacetate is converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK), it will be converted to glucose by hepatocytes. PEPCK is the key gating enzyme of gluconeogenesis. Glucose-6-phosphatase (G6Pase) hydrolyzes glucose 6-phosphate to glucose, thereby releasing it from hepatocytes into the circulation (**Figure 2**). This glucose is utilized for energy by the brain and other organs. Aging decreases the expression of liver and kidney PEPCK and G6Pase and decreases PEPCK levels in the skeletal muscle of mice (20, 26, 120; **Figure 2**). Others have confirmed many of these results (41, 42). These changes are consistent with the decrease in whole-body protein turnover that occurs with age (39, 75).

At the level of enzymatic capacity, the changes in metabolic enzyme expression described above are most likely the result of

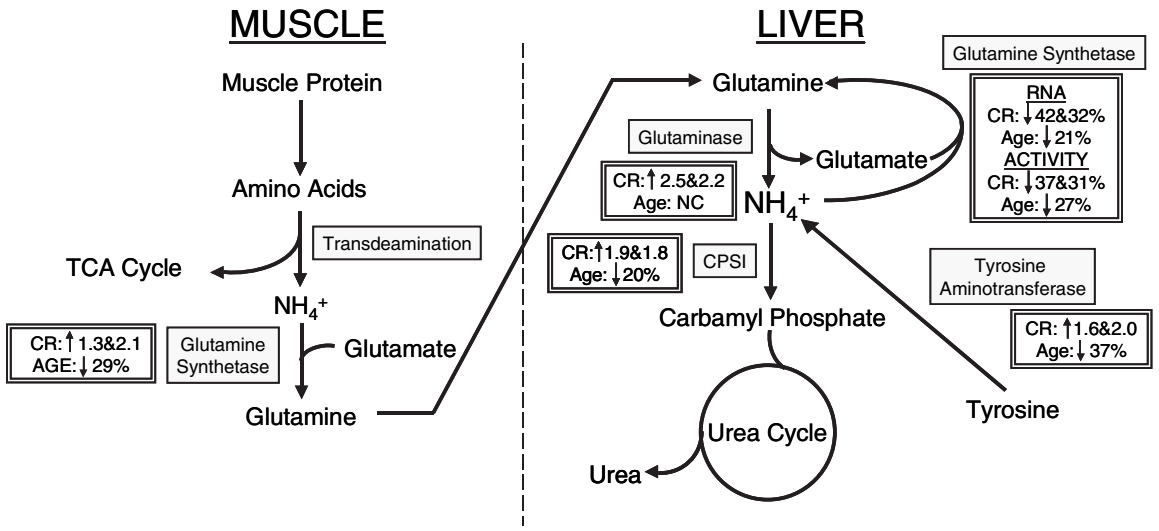


Figure 1

Summary of the effects of age and diet on muscle and liver nitrogen metabolism. In muscle and many other tissues, the degradation of proteins to amino acids is utilized for generating metabolic energy. Transdeamination of amino acids produces tricarboxylic acid cycle intermediates and ammonia. Glutamine synthetase synthesizes glutamine from glutamate and ammonia. Glutamine is transported to the liver in the blood, where glutaminase releases the ammonia, regenerating glutamate. Carbamylphosphate synthase-1 (CPSI) converts this ammonia to carbamylphosphate, which is converted to urea by the urea cycle. The amino group of excess tyrosine is released by tyrosine aminotransferase 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 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.

CR-related adjustments of insulin, glucocorticoid, thyroid hormone, and IGFI levels, and not of changes in substrate availability (27, 124). Thus, the adjustments in enzymatic capacity are a cause rather than an effect of decreased protein turnover during aging (129, 130). Although the studies reviewed focused on the liver and muscle, protein turnover in all or most tissues of the body appears to decline with age (32, 38, 75, 88, 91, 92). Decreased macromolecular turnover may underlie the age-related accumulation of oxidatively damaged protein. This decrease may exacerbate the effects of the oft-reported age-related increase in oxidant production by isolated muscle mitochondria (e.g., 9, 81).

LTCR, STCR, AND AMES DWARFISM: GLUCONEOGENESIS, GLYCOLYSIS, AND LIPOGENESIS

In 1989, Feuers et al. (33) published the first study suggesting that the enzymatic capacity of the liver for gluconeogenesis increases, and the enzymatic capacity for glycolysis decreases, in LTCR mice. Using a variety of biochemical and genic approaches, we were able to confirm and extend these studies (25–27, 124–126). We found that LTCR, STCR, and the Ames dwarf mutation induce the fasting and fed levels of PEPCK and G6Pase activity and/or mRNA in liver (14, 25, 120, 124, 126; **Figures 1** and **2**). G6Pase mRNA is more abundant in LTCR mice, at all times,

even 1.5 hours after feeding (25, 26). STCR and LTCR elevated PEPCK mRNA in both young and old mice (25, 26). PEPCK mRNA and activity decrease within 1.5 hours of feeding, but by 5 hours after feeding, CR mice accumulated twice as much PEPCK mRNA and activity as did control mice (25). LTCR and dwarfism together additively enhanced PEPCK, glucose-6-phosphate isomerase, and glycerol-6-phosphate transporter mRNA (126). The latter two enzymes also have important roles in gluconeogenesis. Thus, LTCR, STCR, and reduced anterior pituitary signaling oppose the age-related decrease in protein turnover by reversing many age-related effects on the activity and/or mRNA of key gluconeogenic enzymes (25–27, 124–126).

Evidence that LTCR enhances protein turnover and utilization for energy production is also found in the expression of nitrogen metabolizing genes. LTCR increases GS expression in muscle and decreases GS activity and mRNA in the liver (**Figure 1**), even 1.5 hours after feeding (25, 26). Dwarfism also reduces hepatic glutamine synthetase expression (126). These effects spare glutamate reutilization for glutamine production, thereby fueling hepatic gluconeogenesis and ureagenesis. LTCR leads to a 2.5-fold increase in hepatic glutaminase mRNA (26; **Figure 1**). The level of this mRNA closely reflects hepatic glutaminase activity (136). CPSI mRNA in young and old CR mice is two to five times its level in control mice (25, 26, 125). CPSI gene transcription responds rapidly to reduced caloric intake, leading to a rapid increase in CPSI mRNA, protein, and activity (125). The initiation of CR also rapidly induces three other urea cycle enzymes, argininosuccinate synthetase 1, argininosuccinate lyase, and arginase 1 (22). Together, these results indicate that CR animals have a significantly enhanced capacity for protein turnover, gluconeogenesis, and nitrogen disposal for energy generation, even soon after feeding. A number of these results have been confirmed by others (33, 41, 66, 77).

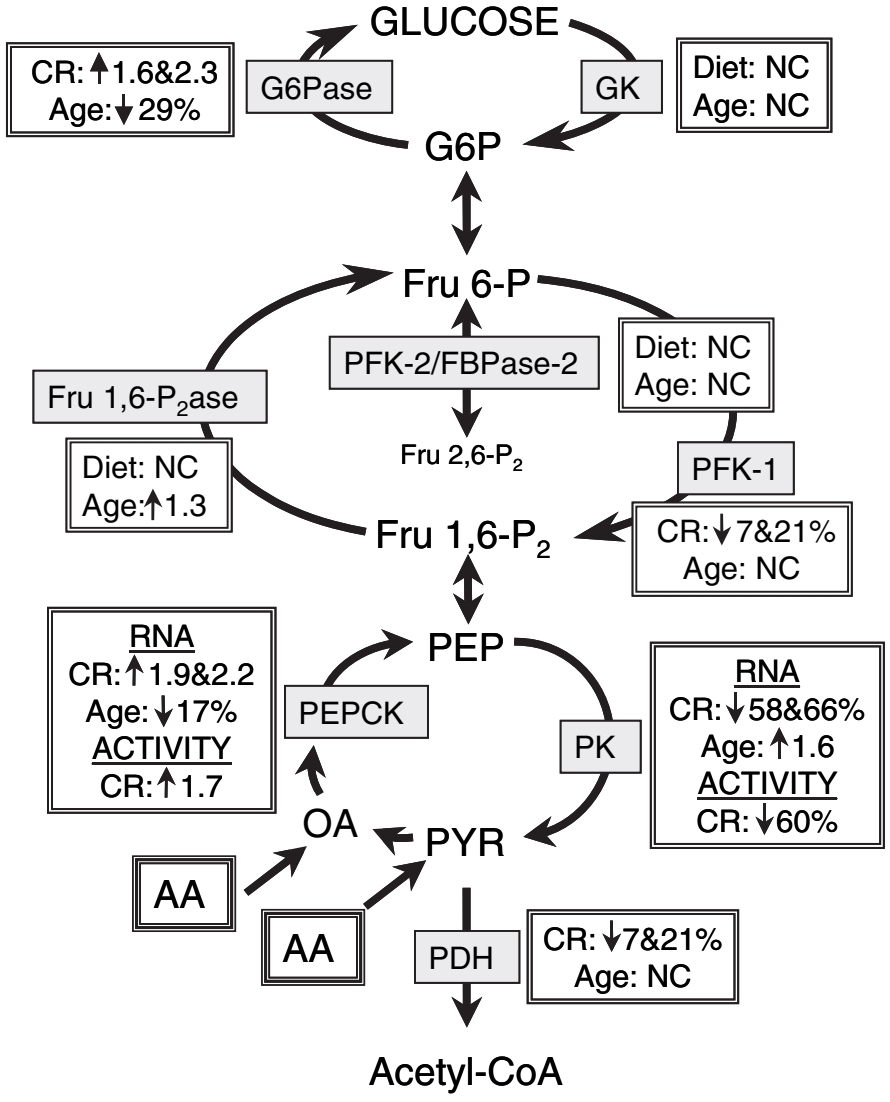
The changes in gene expression discussed above are most likely the result of CR- or dwarfism-related alterations in insulin, glucocorticoid, thyroid hormone, and/or IGFI levels (reviewed in 27, 124). This suggests that CR mice catabolize protein derived from proteolysis, autophagy, and the autophagic and apoptotic death of mitotically competent cells to generate substrates for energy production. CR mice are approximately four times more insulin sensitive than control mice, leading to greatly reduced serum insulin levels (25). Insulin also can be a strongly antiapoptotic comitogen in the liver and other tissues (11). After feeding, increased blood insulin levels drive a compensatory wave of macromolecular biosynthesis and cell division (25). Thereafter, as glucoregulatory hormone levels respond to fasting, carbon and nitrogen from liver and other tissues begin to flow back to the liver for gluconeogenesis and disposal, respectively. In this way, CR appears to drive balanced waves of catabolism and compensatory resynthesis, resulting in both decreased tumor initiation and growth and reduced accumulation of damaged macromolecules. In both LTCR and dwarf mice, these cycles appear to produce smaller mice with smaller organs.

LTCR and Ames dwarf mice also underexpress the key gating enzymes of glycolysis (glucokinase, pyruvate kinase, and acetyl-CoA carboxykinase), which suggests that both decrease substrate availability for de novo liver lipogenesis (25, 126). Both of these longevity enhancers decreased the hepatic expression of 16 lipid- and cholesterol-related genes, including genes involved in lipid, fatty acid, and cholesterol biosynthesis, lipid transport, and HDL metabolism (126). Further, they induce eight genes for key fatty acid β-oxidation enzymes, several of which are additively induced by the combined treatments. LTCR also decreases the mRNA and activity of the key gating enzyme of fatty acid biosynthesis, pyruvate dehydrogenase, even in the hours following feeding (25, 26, 120). Together, these results suggest that two means of lifespan extension

produce a sustained decrease in lipogenesis and enhancement of lipolysis in the liver. Others have confirmed a number of these results (42). As mentioned above, fatty acid biosynthesis appears to be required for the survival of many cancer cells (72, 108).

STRESS RESISTANCE IN POSTMITOTIC CELLS

Although there are similarities in the genic responses of heart and liver to LTCR, the differences may be most apposite to its differential effects on mitotic and postmitotic tissues (**Table 2**). For example, although CR shifted liver toward a genic profile consistent with increased apoptotic cell death, no such shift was found in heart (28). CR is known to suppress apoptotic cell death and increase the stress resistance of the cardiovascular and cerebrovascular systems in vivo (84). Apoptotic or autophagic loss of postmitotic cells in these tissues would be highly disadvantageous, and the oncogenic transformation of



postmitotic cells is rare. Thus, reduced stress and increased stress resistance is closely associated with reduced functional impairment in these organs. An illustration of this can be found in the heart. Aging impairs cardiac capacity, contractility, and diastolic and systolic function (87). Approximately 40% of male C57BL/6 mice develop cardiomyopathy by 1000 days of age (127). In rodents and humans, three major age-associated changes markedly affect myocardial performance. First, myocardial fibrosis, a hallmark of cardiac aging in humans and rats, is initiated by cellular necrosis and apoptosis, which induce reparative interstitial and perivascular collagen deposition (3, 19, 31). Fibrosis decreases cardiac distensibility and increases diastolic pressure, impairing coronary hemodynamics, and lowering coronary reserve (57, 131). Second, there is an age-related decline in the number of cardiomyocytes, which leads to compensatory myocyte hypertrophy (17, 28, 79). Left ventricular hypertrophy is the most common cardiac manifestation of aging (73). Third, aging produces impairment in mitochondrial bioenergetics, which appears to contribute to myocardial stiffness, apoptosis, atrophy, and compensatory hypertrophy (95, 128). These changes may underlie age-related cardiac arrhythmias, dysfunction, and failure. LTCR enhances cardiovascular function and reduces these manifestations of aging (84). LTCR reduces risk factors for coronary artery disease and stroke in humans and other animals (36, 84, 93). Moderate CR improves cardiac remodeling and diastolic dysfunction in the Dahl-SS rat, which develops age-related hypertension-associated diastolic dysfunction (114).

We and others investigated the effects of LTCR on global gene expression in mouse heart (28, 37, 74). We conducted high-density microarray, biochemical, and histochemical studies of STCR and LTCR. Although there are changes in gene expression common to heart and liver (37; **Table 2**), most responses to CR are tissue-specific and tailored to the age-related dysfunctions of each organ. In heart, many downregulated genes are associated with reduced fibrosis, tissue remodeling, and blood pressure. Many of the changes in signal transduction–related gene expression

Figure 2

Summary of the effects of age and caloric restriction (CR) on the 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 C6, yielding glucose 6-phosphate (G6P). The committed step in glycolysis, and the second irreversible and regulated step, is the phosphorylation of fructose 6-phosphate (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 adenosine diphosphate (ADP) are utilized by pyruvate kinase (PK) to produce pyruvate (PYR) and adenosine triphosphate (ATP). Pyruvate dehydrogenase (PDH) oxidatively decarboxylates pyruvate to form acetyl-CoA, which is a bridge between glycolysis and the tricarboxylic acid cycle or fatty acid biosynthesis. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the first committed step in gluconeogenesis. The main noncarbohydrate precursors for gluconeogenesis are amino acids from the diet and from protein breakdown in muscle and other organs. 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 Fru 6-P from 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). In the figure, 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 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.

appear to be focused around the differentiated functions of heart, including enhanced lipid catabolism for energy production and enhanced contractility (28). We found no evidence for enhanced apoptosis-related gene expression in heart, in sharp contrast to the changes found in liver.

In heart, STCR induces a relatively small subset of the LTCR-responsive genes (28). However, these changes appear to be important and consistent with the rapid effects of CR on cardiac physiology. Just four months of CR reduces oxidative, glycoxidative, and lipoxidative damage to rat heart mitochondrial proteins (102). Just eight weeks of CR produces many LTCR-like changes in the expression of most extracellular matrix genes and in many genes related to the cytoskeleton, cell motility, signal transduction, differentiation, cell division, immune function, inflammation, chaperones, and stress (28). When LTCR mice were fed a control diet for just eight weeks, 97% of the LTCR-responsive genes returned to control expression levels (28). Thus, even in heart, gene expression can be rapidly responsive to shifts to and from a CR-associated genic profile.

SHIFTING FROM THE CONTROL TO THE CR STATE

The results reviewed above raise an important question. How rapid and complete is the shift from the control to the CR state when CR is initiated later in life? Another way of asking this question is, Can STCR induce all of the benefits of LTCR? The studies reviewed here suggest at least three categories of changes are induced later in life. First are changes that respond rapidly and completely. These include the rapid genic responses to CR in liver and heart that are reviewed above. Second are the changes that take longer than eight weeks. These include processes like the depletion of perivascular collagen in cardiac vessels (28). Eight weeks of CR in old mice downregulated the expression of many extracellular matrix genes but did not significantly reduce the level of perivascular collagen deposited around cardiac vessels. The turnover of extracellular matrix is relatively slow and probably requires longer than eight weeks to decrease significantly. A third type of LTCR-related change probably cannot be reproduced by late-onset CR. Such changes include the loss of cardiomyocytes in the left ventricle of the heart during aging. This change is unlikely to be altered by late-life CR.

CONCLUSIONS

Metazoan evolution has selected a complex web of physiological changes in response to shortages in nutritional energy. Genomewide microarray and conventional molecular and biochemical studies indicate that aging is accompanied by a genic drift toward gene-expression patterns associated with the characteristic age-related pathologies of specific tissues and toward increased inflammation, cellular stress, and fibrosis. LTCR, STCR, and other means of extending lifespan appear to shift intracellular and extracellular signaling toward a physiologic and genic state that reverses or resists these changes. Although many of the genic changes induced by CR are conserved between functionally different tissues such as liver and heart, most appear to be tailored to organ-specific functions. Broadly, mitotically competent tissues shift toward susceptibility to apoptotic and autophagic cell death, whereas postmitotic tissues enhance stress resistance and cellular repair. Many of the genic responses to CR shared by tissues appear to involve accommodation to reduced macromolecular biosynthesis. Despite these reductions, CR increases the overall rate of macromolecular turnover to mobilize metabolic energy. Evidence for this can be found in the liver, where alterations in the enzymatic capacity for glycolysis, gluconeogenesis, and ureagenesis result in reduced lipid biosynthesis and increased glucose biosynthesis and nitrogen disposal. Together these changes show that lifespan extension is

a multifaceted process derived in part from repeated cycles of cell and tissue turnover in mitotically competent tissues and tissue turnover and repair in postmitotic tissues.

SUMMARY POINTS

1. LTCR produces a subset of genic and physiologic effects common to the mitotic liver and postmitotic heart. These responses suggest that CR produces a widespread reduction in protein, RNA, DNA, and lipid biosynthesis and adjusts the rates of protein degradation, particularly glycoprotein degradation, to compensate.
2. Most of the genic responses to CR are tissue specific and tailored to the age-related physiologic changes and age-related diseases of each tissue.
3. In heart, CR rapidly produces changes in gene expression consistent with reduced fibrosis, remodeling, cytoskeletal dynamics, cell motility, inflammation, immune activation, blood pressure, and protein degradation.
4. In liver, CR produces changes in gene expression consistent with reduced glycolysis and increased gluconeogenesis, ureagenesis, and susceptibility to apoptosis. Many of these changes are enhanced or recapitulated by the Ames longevity mutation.
5. LTCR and STCR initiated late in life suppress the growth rate of tumors, apparently by increasing the rate of apoptosis of tumor cells.
6. The whole-body protein turnover driven by CR can be detected by the enhanced expression of gluconeogenic and ureagenic enzymes in the liver.
7. CR initiated late in life can recapitulate many, but probably not all, of the beneficial effects of LTCR in heart. It can induce gene expression changes consistent with reduced blood pressure and extracellular matrix overexpression and enhanced lipid β-oxidation for energy generation.
8. CR initiated late in life can recapitulate the anticancer and most of the other effects of LTCR.

FUTURE ISSUES

1. Studies need to define how CR enhances the apoptotic potential of tumors and some mitotic tissues while reducing the apoptotic potential of many postmitotic tissues.
2. Studies need to determine how CR induces higher rates of apoptosis in tumors, especially in spontaneous hepatomas. Untreated patients with hepatoma usually die in 3–4 months, whereas treated patients live 6–18 months, if they respond to therapy. Clearly, novel targets are needed.
3. Studies of the gluconeogenic, glycolytic, and nitrogen disposal pathways need to investigate how the changes in the enzymatic capacity of specific steps in these pathways actually change carbon and nitrogen flux through the pathways.
4. Studies need to continue the identification of the signal-transmission pathways altered by CR that lead to its anticancer and cardiovascular benefits.

5. Continuing research is needed to identify pharmacologic agents that target the pathways used by CR to produce its anticancer and other health benefits.
6. The effects on lifespan of mutational or pharmacological manipulation of the pathways of intermediary metabolism need to be conducted to define the scope of their probable effects on lifespan and health span.
7. When studies have progressed sufficiently, potential longevity therapeutics identified in animals should be studied for their effects in humans.

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Errata

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