



Review

Caloric restriction: From soup to nuts

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ABSTRACT

Caloric restriction (CR), reduced protein, methionine, or tryptophan diets; and reduced insulin and/or IGFI intracellular signaling can extend mean and/or maximum lifespan and delay deleterious age-related physiological changes in animals. Mice and flies can shift readily between the control and CR physiological states, even at older ages. Many health benefits are induced by even brief periods of CR in flies, rodents, monkeys, and humans. In humans and nonhuman primates, CR produces most of the physiologic, hematologic, hormonal, and biochemical changes it produces in other animals. In primates, CR provides protection from type 2 diabetes, cardiovascular and cerebral vascular diseases, immunological decline, malignancy, hepatotoxicity, liver fibrosis and failure, sarcopenia, inflammation, and DNA damage. It also enhances muscle mitochondrial biogenesis, affords neuroprotection; and extends mean and maximum lifespan. CR rapidly induces antineoplastic effects in mice. Most claims of lifespan extension in rodents by drugs or nutrients are confounded by CR effects. Transcription factors and co-activators involved in the regulation of mitochondrial biogenesis and energy metabolism, including SirT1, PGC-1 α , AMPK and TOR may be involved in the lifespan effects of CR. Paradoxically, low body weight in middle aged and elderly humans is associated with increased mortality. Thus, enhancement of human longevity may require pharmaceutical interventions.

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1. Introduction

Humans have been extending their average lifespans linearly since about 1840 (Oeppen and Vaupel, 2002). By 1900, the average lifespan in Europe and the USA had increased from between 22 and 35 years, to about 60 years of age. Today, the average lifespan world wide has risen to roughly 63 years (Oeppen and Vaupel, 2002). In Japan, where the longest lifespans are currently found, the life expectancy for women is almost 85 years (Oeppen and Vaupel, 2002). Because of these gains, by the 1960s, the human survival curve in developed countries began to resemble that of research animals protected in a vivarium. Because it is mostly the old who die, people born today have a better chance of reaching extreme old age than do the current "old". For example, the life expectancy of living to 100 years for boys and girls born today in the United Kingdom is 18.1% and 23.5%, respectively (Anonymous, 2007). In contrast, a 40-year-old man and woman have only an 8% and 11.7% chance of reaching 100 years of age, respectively. As of May 26, 2008, the Los Angeles Gerontology Research Group had verified only 74 living supercentenarians (people living to 110 years of age or above) [64 females and 10 males (<http://www.grg.org/Adams/Tables.htm>)]. Only one human, Mme.

Jeanne Calment, is credibly documented to have survived to 122 years of age.

Demographers estimate from the shape of lifespan curves in developed countries that humans are approaching the theoretical lifespan limits of our species (Olshansky et al., 2001). It has been estimated that if we successfully conquer all the diseases that currently kill us, including cancer and cardiovascular disease, that are the major killers in industrialized societies, we will extend our average lifespan by only about 15 years (Olshansky et al., 2001). Thus, even if we are lucky enough to escape all the diseases currently killing us, we will still die when we encounter the "wall" of our maximum lifespan, looming at approximately 110 years of age. Maximum lifespan is often defined as the lifespan of the longest lived 10% of a cohort. The issues surrounding our maximum lifespan are part and parcel of the health care dilemma facing the developed world. A major portion of the spending for health-related research and care by individuals, governments, private companies and foundations, doctors, hospitals, and other health care providers are focused on capturing that final 15 years of life. But, perhaps this need not be the case.

2. Lifespan effects of caloric restriction (CR)

Scientists have known since the 1930s that diets which reduce calories below the level required for maximum fertility and

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fecundity, while avoiding malnutrition, can extend the mean and maximum lifespan of laboratory rats, by 40% or more (McCay et al., 1935). Such dietary regimens are often termed “CR” or “dietary restriction”. Early reports also showed that CR reduces the incidence and severity of many of the diseases which limit the lifespan of rats (McCay et al., 1935). Subsequent investigations confirmed that CR extends survival, reduces the incidence of tumors and other diseases, and shifts the onset of these diseases to later ages in laboratory rodents (Cheney et al., 1980; Weindruch and Walford, 1988; Masoro, 2006).

2.1. Phylogenetic conservation of the CR response

The longevity and health effects of CR appear to be widespread phylogenetically. As summarized by Masoro (2006), they include species from three Kingdoms (Animalia, Fungi, and Protocista), and four phyla within Animalia (*Rotifera*, *Nematoda*, *Arthropoda*, and *Chordata*). Responsive species include dog (Labrador Retriever) (Lawler et al., 2008), rodents (rats, mice, hamsters) (Weindruch and Walford, 1988), nematode [*Caenorhabditis elegans* (*C. elegans*)] (Klass, 1977), rotifer (*Asplanchna brightwelli* and *Philodena acuticornis*) (Fanestil and Barrows, 1965; Verdone-Smith and Enesco, 1982), spider (*Frontinella pyramitela*; a.k.a. bowl and doily spider) (Austad, 1989), fruitfly [*Drosophila melanogaster* (*Drosophila*)] (Min et al., 2007), the Mediterranean fruit fly (*Ceratitis capitata*; a.k.a. medfly) (Carey et al., 2005), guppies (*Lebistes reticulatus*, Peters) (Comfort, 1963), and zebrafish (*Danio rerio*) (Keller et al., 2006). Nutritional insufficiency also appears to extend the chronological and replicative lifespan of baker's yeast (*Saccharomyces cerevisiae*), although the meaning of these observations and their relationship to the lifespan of multicellular eukaryotes remains unclear (Fabrizio and Longo, 2007; Kaeberlein and Powers, 2007; Michan and Sinclair, 2007).

Recently, gerontologists have become more sensitive to the idea that the apparent “universality” of the CR response could be the result of reporting bias. The health and/or longevity effects of CR may not be universal, even within species. For example, while CR begun at 4 months of age increases median and maximum lifespan of C57BL/6 and B6D2F1¹ mice, it fails to alter the lifespan of DBA/2 mice, at least using the methods of these studies (Forster et al., 2003). A CR regimen has not been described that is capable of extending the lifespan of the housefly (*Musca domestica*) (Cooper et al., 2004). In addition, some age-related diseases, such as certain mouse models of Parkinson's disease, also may be unresponsive to CR (Armentero et al., 2008).

These data may not indicate that CR cannot extend the lifespan of these species or strains. Responsiveness to CR can depend on subtleties of the treatment protocol. CR was long thought incapable of extending the lifespan of middle-aged and older mice. Only when CR was introduced in a stepwise fashion was lifespan extension obtained in mice 12 months of age or older (Weindruch and Walford, 1982; Dhahbi et al., 2004). Harrison and Archer found a shortening of lifespan when CR was introduced to C57BL/6J mice at 4 weeks of age, immediately after weaning (Harrison and Archer, 1987). However, other laboratories obtained robust lifespan extension with this mouse strain when CR was initiated in middle age (e.g. Pugh et al., 1999). Initial reports

indicated that medflies were unresponsive to CR (Carey et al., 2002). However, clear evidence of a CR response was found when protocols similar to those used for *Drosophila* were employed (Carey et al., 2005; Davies et al., 2005).

Triggering the CR response in some strains or species may require specific husbandry techniques and/or dietary regimen. Differences in the husbandry of some strains of dwarf mice determines whether shortened or lengthened lifespan is obtained relative to controls (Bartke, 2008b). One interpretation of such results is that certain stresses [e.g. CR; reduced insulin and insulin-like growth factor-I (IGFI) signaling] induce a repair and survival-related physiological response. For lifespan extension to be observed, the response must obviate the negative effects of the inducer and redirect molecular priorities to pathways designed for stress resistance, repair, and survival (Schumacher et al., 2008). Whether a given stress produces extended longevity in a specific strain or species will depend on the severity of the stress, the degree to which it induces the response pathway, and the potency of the response pathway. These are all polygenic traits. Thus, it is not surprising that genetic background can have significant influences on the response.

2.1.1. CR in nonhuman primates

Studies from two colonies of rhesus macaques suggest that the effects of CR in nonhuman primates recapitulate many of the physiological, hematological, hormonal, immunological, and biochemical effects produced in rodents (Kemnitz et al., 1993; Mattison et al., 2003, 2005; Maswood et al., 2004; Roth et al., 2004; Anderson et al., 2009). Approximately 30% CR (a 30% reduction in calories) initiated in macaques of various ages, decreases body weight and adiposity (Colman et al., 1998; Lane et al., 1999); improves glucoregulatory functions and increases insulin sensitivity (Lane et al., 1995, 1999; Gresl et al., 2003); produces favorable changes in blood triglyceride and lipid profiles (Edwards et al., 1998); reduces serum levels of C-reactive protein (Edwards et al., 1998); delays male skeletal and sexual maturation; delays the age-associated decline in serum dehydroepiandrosterone and melatonin normally found in ad libitum fed controls (Lane et al., 1999; Mattison et al., 2003); reduces oxidative damage in skeletal muscle (Zainal et al., 2000); and attenuates the development of sarcopenia (Colman et al., 2008). Long-term CR (LTCR) also produces a trend toward reduced cardiovascular disease, diabetes, neoplastic disease, and liver failure as causes of mortality (Lane et al., 1999; Roth et al., 1999). LTCR refers to CR initiated when an animal is young and maintained throughout most or all of its remaining lifespan. CR monkeys develop diabetes later in life and with a lower incidence than ad libitum fed controls (Bodkin et al., 1995). CR initiated during adulthood may delay T-cell aging and preserve naïve CD8 and CD4 T cells into advanced age, although the timing and method of introduction of CR appears to be crucial to this effect (Messaudi et al., 2008). LTCR also reduces the production of local inflammatory mediators and the risk of inflammatory periodontal disease among male macaques (Reynolds et al., 2009).

Results from a third colony of rhesus macaques have been interpreted as evidence that CR extends the lifespan of these primates (Bodkin et al., 2003). However, statistical and methodological concerns make these conclusions equivocal (Lane et al., 2004). Recently, investigators at the Wisconsin National Primate Research Center have published an analysis of their survival data that found a statistically significant increase in the lifespan of CR rhesus macaques (Colman et al., 2009). However, their posthoc data analysis excludes deaths deemed not “age related”. This analysis did not use a multiple testing procedure to compensate for this post hoc design. Further survival data from the two ongoing studies will be needed to conclusively determine whether CR is effective at extending the lifespan of nonhuman primates.

¹ Hybrid (F1) rodents are produced by crossing mice of two different inbred strains. Although they are heterozygous at all loci for which their parents have different alleles, they are genetically and phenotypically uniform. The standard nomenclature lists the female parent first and the male parent second, separated by an “×”. For some F1 rodent strains a single name is used which is derived from an abbreviation for the female parent strain (listed first) and an abbreviation for the male parent strain (listed second) followed by F1. For example, a cross between a female C57BL/6 and a male DBA/2 mouse can be designated as a “C57BL/6 × DBA/2” or as a “B6D2F1” strain mouse.

2.1.2. CR in humans

Some have argued that it is unlikely that CR or any other treatment can prolong human health- and lifespan due the age-related loss of molecular fidelity resulting from the inevitable increase in entropy (Hayflick, 2004). Others have argued that the life-history of humans presents little selective pressure for a robust CR response (Phelan and Rose, 2005; Demetrius, 2005; De Grey, 2005). However, as with the nonhuman primates discussed above, it is unlikely that we will have an unequivocal resolution of this debate in the near future.

The limited evidence available for humans suggests that CR produces physiological effects that are similar to those found in rodents and monkeys (Verdery and Walford, 1998; Walford et al., 1999, 2002; Weyer et al., 2000). However, there are few studies in the literature of human health and longevity using nutritious, low calorie diets. Kagawa reported that in the 1970s the death rates from cerebral vascular disease, malignancy, and heart disease on Okinawa Island were 59%, 69%, and 59% of those found in the rest of Japan (Kagawa, 1978). The mortality rate for 60–64 year olds living on Okinawa was half of that found elsewhere in Japan. The incidence of centenarians on the island was two to forty times greater than that of other Japanese communities. He suggested this good fortune resulted from CR. Okinawan school children consumed only 62% of the calories of other Japanese school children during the early 1960s (Hokama et al., 1967). Kagawa reported in 1978 that Okinawan adults consumed 20% fewer calories relative to the national average in Japan (Kagawa, 1978). Okinawans who emigrated to the USA and began to consume a more typical Western diet had mortality and morbidity rates similar to others in the USA (Kagawa, 1978). The conclusions of this study are supported by subsequent studies using six decades of archived population data on elderly Okinawans (aged 65 years and older) regarding diet composition, energy intake, energy expenditure, anthropometry, plasma DHEA, mortality from age-related diseases, and current survival patterns (Willcox et al., 2007). Willcox et al. (2007) found low caloric intake and negative energy balance, little weight gain with age, life-long low BMI, higher plasma DHEA levels during aging, low risk of mortality from age related diseases, and survival patterns consistent with extended mean and maximum lifespan in this cohort.

A role of CR in human health is also supported by a study of 60 healthy seniors (average age of 72 years at the start of the study) who received a dietary regimen averaging 1500 kcal/day for 3 years, versus an equal number of controls consuming 2300 kcal/day (Vallejo, 1957). The CR group consumed 2300 kcal/day every-other-day, and one liter of milk and 500 g of fruit on the alternate day. Analysis of these data indicates that CR significantly lowered the rates of hospital admissions (123 versus 219 days; $p < 0.001$) and numerically lowered deaths (6 versus 13) (Stunkard, 1976).

A longitudinal CR study conducted by Walford and colleagues on eight healthy nonobese humans eating a low-calorie nutrient-dense diet for 2 years in Biosphere 2 (1750–2100 kcal/day), found 50 CR-related changes in physiologic, hematologic, hormonal, and biochemical parameters that resemble those of CR rodents and monkeys (Walford et al., 1999, 2002; Weyer et al., 2000).

More recently, a number of studies have been performed on groups of volunteers subjected to CR for 6 months or one year. Studies of a group of individuals consuming a nutrient-rich, low-calorie diet for an average of 6 years (BMI 19.6 ± 1.9) and age-matched healthy controls eating typical American diets (BMI 25.9 ± 3.2) found that the CR group had markedly reduced serum total cholesterol, low-density lipoprotein cholesterol, ratio of total cholesterol to high-density lipoprotein cholesterol, triglycerides, fasting glucose, fasting insulin, C-reactive protein, platelet-derived growth factor AB, and systolic and diastolic blood pressure (Fontana et al., 2004). Carotid artery intima-media thickness was ~40% lower

in the CR group. High-density lipoprotein cholesterol was higher with CR. Another study of healthy, middle aged subjects practicing CR, this time for an average of 6.5 years, found lower heart chamber viscoelasticity and stiffness, lower blood pressure, and lower serum C-reactive protein, tumor necrosis factor- α , and transforming growth factor- β 1 levels than were found in age- and gender-matched controls consuming Western diets (Meyer et al., 2006). Together, these studies suggest that longer-term CR initiated in older humans reduces blood pressure, systemic inflammation, myocardial fibrosis, and other risk factors for cardiovascular disease.

Studies resulting from a 3 center, NIA sponsored investigation of human CR, termed CALERIE (Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy) investigated the cardiovascular effects of CR and exercise in a one year controlled study of sedentary, nonobese middle-aged men and women randomly assigned to a 20% CR diet, an exercise regimen that increased energy expenditure by 20% with no compensatory increase in energy intake, and controls given only guidelines for a healthy lifestyle. The study found that both CR and exercise produced similar reductions in coronary heart disease risk factors, including adipose mass, plasma LDL-cholesterol, total cholesterol/HDL ratio, homeostasis model assessment of insulin resistance index, and serum C-reactive protein levels (Fontana et al., 2007). A similar 6 month study using other human volunteers reported very similar results (Lefevre et al., 2009).

Human CR appears to have effects on sex steroids, insulin, and IGF1 that are similar, but not identical, to those found in rhesus monkeys and rodents. A cross-sectional comparison of individuals eating a low protein CR diet for an average of 4.4 years (mean BMI 21.3), endurance runners (mean BMI 21.6), and age- and sex-matched sedentary controls eating Western diets (mean BMI 26.5) found that especially the low-protein and CR group, but also the runners, had significantly lower plasma levels of insulin, free sex hormones, leptin, and C-reactive protein, and higher sex hormone-binding globulin than the control group (Fontana et al., 2006a). A one year controlled study of sedentary, nonobese middle-aged men and women randomly assigned one year of exercise training, CR, or a healthy lifestyle control group, found that weight loss induced by either means produced improvement in glucose tolerance and insulin action (Weiss et al., 2006). A low-protein, CR diet also reduced plasma IGF1 and the ratio of IGF1 to insulin-like growth factor binding protein-3 (IGFBP-3²) more than endurance running (Fontana et al., 2006a). Thus, a low-protein, CR diet reduces risk factors for atherosclerosis, and growth factors and hormones linked to an increased risk of cancer. Interestingly, CR without a decrease in protein intake did not reduce serum IGF1 levels in humans (Fontana et al., 2006a, 2008). This dependence of IGF1 levels on protein intake is likely the reason that the Biosphere and CALERIE studies, which had higher levels of dietary protein, did not detect a decrease in serum IGF1 levels (Walford et al., 2002; Holloszy and Fontana, 2007; Redman and Ravussin, 2009).

In a weight loss study, a group of overweight male and female volunteers (mean BMI of 27.5) were subjected to 25% CR diets and a combined diet and exercise regimen for 6 months (ending BMI of 24.8). The CR diets and combined diet reduced fasting insulin levels, but not DHEAS or glucose levels. Core body temperature and sedentary 24-h energy expenditure decreased in all the CR groups (Heilbronn et al., 2006). This decrease may be due to the CR-related decrease in serum T₃ concentrations found by Fontana and

² IGFBP-3 is the most abundant member of the IGFBP family in serum. It binds and sequesters IGF1 and IGFII in a circulating complex, which inhibits their interaction with the IGFIR, thereby reducing their mitogenic and anti-apoptotic activities. While IGFBP-3 is generally regarded as an inhibitor of IGF1 action, under some circumstances it can enhance the activity of IGF1 by increasing its bioavailability.

Holloszy in lean and weight-stable healthy humans consuming a nutrient dense, CR diet for 3–15 years (Fontana et al., 2006b). The decrease is also similar to that found in CR rodents. A decrease in DNA damage and an increase in muscle mitochondrial DNA and the expression of genes encoding proteins involved in mitochondrial function has also been reported in muscle biopsies of humans subjected to 6 months of CR or CR and exercise (Heilbronn et al., 2006; Civitarese et al., 2007).

Clear downsides have been identified for CR in humans in addition to the decrease in free sex hormones and increase in sex hormone-binding globulin mentioned above. Twelve months of CR or exercise initiated in healthy middle aged men and women (BMI 23.5–29.9) led to a loss in lean body mass in both groups, but loss of more thigh muscle volume and composite knee flexion strength in the CR group relative to the exercisers (Weiss et al., 2007). VO_2 max also decreased in the CR group, but increased in the exercisers. One year of CR in humans also decreased bone mineral density at the total hip, intertrochanter, and spine of the CR group but not for an age and sex matched group of the exercisers (Villareal et al., 2006). Thus, CR-induced weight loss reduces muscle mass and physical work capacity, and bone mineral density at clinically important fracture sites.

Despite the results suggesting that CR can lead to lifespan extension in humans, this conclusion is not supported by prospective cohort studies of the relationship between BMI and longevity in humans (see below). These studies and their possible meanings are discussed later in this review.

2.1.3. Age and CR responsiveness

Older mice can respond to CR when it is incrementally introduced (Dhahbi et al., 2004). Ad libitum fed, male, C3B6F1 mice, are poised to begin the accelerated mortality phase of their lifespan curves at about 19 months of age. If they are shifted to a CR diet at that time, they rapidly assume a new mortality trajectory which is characterized by fewer cancer-related deaths, and an increase in both mean and maximum lifespan (Spindler, 2005). In these mice, the decrease in mortality results almost entirely from reduced rates of tumor-associated deaths. The shift in lifespan is accompanied by a concomitant change in the global patterns of gene expression in the mice, especially in the liver, to a pattern which recapitulates most key features of life-long caloric restriction (Dhahbi et al., 2004; Spindler, 2005). Likewise, shifting mice from a life-long CR diet to a control diet at an older age rapidly reverts most CR-specific gene expression in the heart and liver back to the expression levels found in control fed mice (Dhahbi et al., 2004, 2006). Thus, some strains of mice appear capable of rapidly shifting to a CR or control physiological state, even at advanced ages. Whether such shifts occur at advanced ages for all strains of mice, or for mice older than 19 months of age is not known.

Rats may be less responsive to CR initiated at advanced ages. Restriction of old Long Evans (18 months old) and F344 × BN F1 hybrid rats (18 and 26 month old) to approximately one third fewer calories than their control fed littermates produced no increase in longevity (Lipman et al., 1995; Lipman et al., 1998). Interpretation of these studies is confounded by the absence of a positive control. No animals in these studies achieved lifespan extension. Thus, it remains possible that the rats were simply refractory to CR under the conditions used.

Short-term CR (STCR) in middle age or older mice and rats improves protein turnover, upregulates proteasome activity in liver and skeletal muscle, decreases protein carbonyls in liver mitochondria and skeletal muscle, reduces oxidative DNA damage, and increases carbonyl modifications in histones to levels found in young animals (Goto et al., 2007). These results suggest that STCR in middle aged and old rodents produces a wide spectrum of positive effects on protein metabolism.

2.1.4. CR intensity and the lifespan response

The effects of CR on lifespan appear to be dose-responsive. In a compilation of 24 published survival studies, the increase in survival appears to be inversely proportional to the decrease in calories (Merry, 2002). The gene-expression effects of CR also are dose responsive. Caloric restriction at 20% or 50% of ad libitum intake proportionately increased insulin receptor (IR) mRNA and decreased GRP78 (BiP) and GRP94 mRNA in young, middle-aged, and old mice (Spindler et al., 1990, 1991).

A frequent confound to such studies is the issue of food consumption by the control group. Ad libitum feeding is often used for the control group. However, ad libitum intake can be strongly influenced by the caloric density, palatability, and physical form of the food. We have observed that mice fed Purina laboratory chow ad libitum tend to be leaner, probably from eating fewer calories, than mice fed hard packed AIN-93M diet (unpublished observations). Unfortunately, the number of calories consumed by the ad libitum group is seldom measured or reported.

2.1.5. CR duration and the lifespan response

Walford and colleagues reported that the effects of CR on lifespan are proportional to the length of time on CR, whether the CR period was before weaning, after weaning, early in life, or after 15 months of age (Cheney et al., 1983). Data compiled from 21 independent studies confirms that the duration of CR is directly proportional to the increase in longevity in rodents, irrespective of when CR is begun (Merry, 2002). To a first approximation, this relationship appears to be linear, irrespective of the period of life during which CR is administered (however, see “Does CR induce a physiological memory”, below and “Age and CR responsiveness”, above) (Weindruch and Walford, 1982; Cheney et al., 1983; Yu et al., 1985; Beauchene et al., 1986; Merry, 2002; Dhahbi et al., 2004).

2.1.6. Rapidity and reversibility of inducing the “CR state”

The most widely accepted evolutionary explanation for the existence of the CR response holds that it evolved early in metazoans as an adaptation to boom and bust cycles in the food supply (see below). A key element of this theory is the idea that animals should be capable of rapidly switching between the CR and the ad libitum fed state. However, there is relatively little evidence that this switching occurs. We addressed this question by shifting a cohort of 19 month old, male, B6C3F1 mice from life-long control feeding to CR (Dhahbi et al., 2004; Spindler, 2005). The accelerated mortality phase of the lifespan curve begins soon after 19 months of age with this mouse strain. Linear-regression and breakpoint analysis were used to estimate the length of time required to initiate lifespan extension (Spindler, 2005). The lifespan effects of CR appeared to begin within 8 weeks of its initiation. As mentioned earlier, the extension of lifespan appeared to be almost entirely due to reduced tumor-related mortality. Because hepatocellular carcinoma is the major cause of mortality in this strain of mice, later-life CR appears to strongly reduce the growth rate of these tumors. In unpublished studies, we found that preexisting liver tumors shrink to approximately half their size 8 weeks after the introduction of CR. This reduction in tumor size does not appear to involve a reduction in cell division rate (unpublished results). Thus, late-life CR may increase rates of apoptosis in preexisting liver tumors.

Importantly, the rate of response to CR appears to be organ-specific. In eight weeks, heart gene expression does not shift as completely from the control to the CR state. Eight weeks after shifting control mice to CR, relatively few of the changes in cardiac gene expression found in LTCR mice were produced (Dhahbi et al., 2006). These results are similar to those found in the white adipose tissue of mice (Higami et al., 2004). Only a few LTCR-responsive

transcripts were differentially expressed 23 days after a shift to CR. Thus, in heart and adipose, CR-related gene expression is induced more slowly and/or less completely after the commencement of CR. Changes in cardiac physiology, such as reduction of perivascular extracellular matrix, are only slowly reversible after the introduction of CR (Dhahbi et al., 2006). Further, age-related cardiomyocyte loss and hypertrophy in the left ventricle probably cannot be reversed by the introduction of CR in older mice (Dhahbi et al., 2006). Therefore, not all of the responses to CR are as rapid or as inducible late in life as its effects on tumor growth.

Using gene-expression as a surrogate for the CR-related physiological state, we attempted to quantify how rapidly the physiological effects of CR are reversed after the cessation of CR. Switching old LTCR mice to control feeding was accompanied by an almost complete shift of the LTCR-responsive genes in the liver and heart to control gene-expression levels within 8 weeks (Cao et al., 2001; Dhahbi et al., 2004; Spindler, 2005). These results suggest that mice can shift rapidly from the CR to the control state.

As indicated above, the rapidity and reversibility of the CR state are consistent with the prevailing theory for the adaptive value of CR. It is also consistent with studies performed in *Drosophila* showing that the short-term rate of mortality (which determines lifespan in this species) is rapidly responsive to dietary calories (Mair et al., 2003). Shifting flies from a control to a CR diet decelerated their short-term risk of death within 2 days, while switching from CR to control had the reverse effect, also within 2 days. These data from flies and mice suggest that the effects of CR are either phylogenetically conserved or result from convergent evolution (Mair et al., 2005). In either case, the rapidity and reversibility of the CR response are adaptive and integral to its physiological role.

2.1.7. Does CR induce a physiological “memory”?

The results cited above suggest that an interval of CR in young rodents can produce a “memory” which persists during subsequent control feeding to extend lifespan (reviewed by Klebanov, 2007). In one such study, CD rats subjected to CR between 21 and 70 days of age had increased life expectancy over rats fed ad libitum throughout their life (Ross, 1972). In another study, Fischer 344 rats fed a 40% CR diet from 1.5 to 6 months of age followed by ad libitum feeding for the remainder of their life had modestly extended lifespan (Maeda et al., 1985; Yu et al., 1985). Numerically similar, but not statistically significant, results have been reported for other rat strains (Nolen, 1972; Ross et al., 1993; Merry et al., 2008). In mice, Walford and colleagues found that preweaning CR, achieved by reducing the opportunity for suckling, followed by lifelong control feeding, consistently produced a numerically longer mean and/or maximum lifespan in both B10C3F1 and C57BL/6J mice (Weindruch et al., 1979; Cheney et al., 1980, 1983).

An important caveat to the interpretation of the studies cited above is that the early CR results in a smaller rodent, which consumes less food throughout the remainder of its lifespan, thereby inducing mild CR and a subsequent increase in lifespan (e.g. Cheney et al., 1980). Walford and his colleagues noted that in their studies and those of Widdowson, a preweaning period of CR led to both a slight increase in lifespan and a decrease in average body weight throughout the remainder of life (Widdowson, 1964; Cheney et al., 1980). This effect may lead to results such as those reported by Grasl-Kraupp, who found that three months of 40% CR early in life resulted in resistance to nafenopin-induced tumorigenesis during a subsequent 9 month period of ad libitum feeding (Grasl-Kraupp et al., 1994). Neither food intake nor weights were reported. Thus, it is highly likely that this memory effect involves an ongoing low level of CR, and not a more mysterious and persistent physiological change.

2.2. Dietary composition, meal frequency, and lifespan

Balanced diets with altered proportions of fat, protein, carbohydrate, or minerals do not alter the lifespan of rats, provided no nutrient is limiting. In general, lifespan is robustly extended when the number of calories consumed is reduced in these balanced diets (Ross and Bras, 1973; Iwasaki et al., 1988; Masoro, 1990). However, severe restriction of dietary protein or specific amino acids also can extend the lifespan of rodents, independently of caloric intake (Pamplona and Barja, 2006). Protein to carbohydrate ratio can also differentially affect the distribution of tumors in ad libitum fed and CR rats (Ross and Bras, 1973). Protein to carbohydrate ratio also can affect the health and longevity of *Drosophila* (Mair et al., 2005; Lee et al., 2008; Skorupa et al., 2008).

2.2.1. Protein restriction

While some have reported that the restriction of dietary protein in the absence of CR has a negative effect on survival (Davis et al., 1983), most studies in rodents report that protein restriction enhances longevity, irrespective of caloric intake (Pamplona and Barja, 2006). Pamplona and Barja compiled eleven published studies of the relationship between protein restriction and lifespan in rats and mice (Pamplona and Barja, 2006). Ten of the eleven studies in their survey found that reductions in dietary protein increase maximum lifespan by an overall average of ~20%. They point out that this extension appears to be about half that frequently reported for LTCR.

Protein restriction shares many of the highly pleiotropic health and physiological effects of CR in addition to its extension of lifespan. These effects include delayed puberty; decreased growth; transiently decreased metabolic rate; preserved cell-mediated immunity; reduced oxidative damage to proteins (Youngman et al., 1992); reduced glomerulosclerosis in mice (Doi et al., 2001), reduced nephropathy and cardiomyopathy in rats (Maeda et al., 1985); enhanced hepatic resistance to toxic and oncogenic insult (Rodrigues et al., 1991), and decreased preneoplastic lesions and tumors.

In *Drosophila*, protein restriction (achieved by a reduction in the concentration of yeast in the diet) has a greater effect on lifespan than the restriction of dietary carbohydrate (achieved by a reduction in the concentration of carbohydrate) (Mair et al., 2005). However, such studies in *Drosophila* are confounded by uncertainties about the relationship between food dilution and caloric intake (Carvalho et al., 2005; Mair et al., 2005; Min et al., 2007). Further, in *Drosophila*, olfaction appears to be a key method of sensing nutrient availability (Libert et al., 2007). The odor of yeast can partially reverse lifespan extension by food dilution-related CR (Libert et al., 2007). Thus, food dilution may involve determinants of lifespan distinct from an actual reduction in calories consumed.

Serum IGFI levels are reduced in rodents by either CR or protein restriction. The IGFI signaling pathway and its homologues are regulators of longevity in *C. elegans*, *Drosophila*, and mice (Kenyon, 2005; Russell and Kahn, 2007; Bartke, 2008a). However, in humans, 1 to 6 years of CR without protein restriction had no effect on total or free serum IGFI levels or the IGFI to IGFBP-3 ratios (Fontana et al., 2008). In contrast, CR with accompanying mild protein restriction did reduce serum IGFI levels and the IGFI to IGFBP-3 ratios (Fontana et al., 2008). Thus, reduced protein intake may be required for CR-mediated lowering of IGFI levels in humans. Partial loss-of-function mutations in the IGFI receptor (IGFR) gene are overrepresented among centenarians compared with controls, suggesting IGFI signaling is involved in human longevity (Suh et al., 2008). Reduced tumor-associated mortality should be one benefit of reduced IGF-I signaling (see Sections 2.7–2.7.2, below). Therefore, lower protein diets may be important for the potential longevity benefits of CR in humans.

Long-term CR in adult humans also decreases serum T₃, a powerful mitogen for some cell types (Fontana et al., 2006b). This may be another mechanism by which CR reduces basal metabolic rate and core body temperature in humans, and thereby reduces cancer incidence and tumor growth in rodents.

2.2.2. Tryptophan restriction

In rodents, restriction of specific amino acids can extend longevity. Thirty and 40% reductions in dietary tryptophan produce elevated initial mortality, but delayed later-life mortality in the survivors, leading to an increase in maximum lifespan (from 30.5 months in control rats to 36.3 months in tryptophan restricted rats) (Segall and Timiras, 1976; Segall, 1979; Timiras et al., 1984). This does not appear to be a simple CR effect, since the tryptophan restricted rats and mice consume slightly more food than controls (De Marte and Enesco, 1986).

Low tryptophan diets appear to decrease multiple biomarkers of aging in rats and mice. These diets delay reproductive senescence (Segall and Timiras, 1976; Segall et al., 1983), senescent-related deterioration of the coat in female rats (Segall and Timiras, 1976), age-related tumor onset in rats and mice (Segall and Timiras, 1976; De Marte and Enesco, 1986), and senescence-associated loss of temperature homeostasis in rats (Segall and Timiras, 1975). For example, female rats fed a low tryptophan diet from 3 weeks of age and returned to a control diet at older ages were capable of producing offspring at 28 months of age (Segall and Timiras, 1976). No control fed rats were capable of having litters after 17 months of age. Tryptophan restricted mice also enjoy a slight (~10%) numerical increase in lifespan at most ages, although it is unclear whether this increase is statistically significant (De Marte and Enesco, 1986).

The initial increase in mortality after the introduction of low tryptophan diets is reminiscent of the early mortality reported for dwarf mice (Fabris et al., 1971; Shire, 1973) and older mice abruptly shifted to CR in old age (Weindruch and Walford, 1988). In these cases, elevated mortality appears to result from animal husbandry. Caging of dwarf mice with retired breeders led to extended, rather than shortened lifespans, as did a more gradual introduction of CR in older mice (Weindruch and Walford, 1982; and see below).

2.2.3. Methionine restriction

Restriction of dietary methionine by 80% also reproduces many of the physiological effects of CR. The effects of methionine restriction are robust. It has extended lifespan in four strains of rats and one mouse strain (Orentreich et al., 1993; Miller et al., 2005; Komninou et al., 2006; Malloy et al., 2006; Pamplona and Barja, 2006). Like CR, methionine restriction produces highly pleiotropic effects on the health and physiology of rodents. Methionine restriction preserves insulin sensitivity; decreases preneoplastic, aberrant crypt foci induced by azoxymethane treatment; prevents age-associated increases in serum lipids; reduces visceral fat mass, IGFI, basal insulin, glucose, and leptin levels; and increases serum adiponectin levels (Malloy et al., 2006). Methionine restriction produces these effects without an apparent reduction in calorie intake (Malloy et al., 2006). Disrupted IGFI signaling produces many of these same effects on physiology and lifespan (Bartke, 2005).

2.2.4. Mechanisms of lifespan extension by protein, methionine, and tryptophan restriction

Relatively little is known about the molecular mechanisms for the physiological effects of protein or specific amino acid restriction. Methionine restriction, CR, and mutations that disrupt GH signaling reduce the blood levels of insulin, glucose (Masoro et al., 1992; Dhahbi et al., 2001), IGFI (Oster et al., 1995; Sonntag

et al., 1999), and thyroid hormones (Weindruch and Walford, 1988) relative to the appropriate control groups (Miller et al., 2005; Bartke et al., 2008). Decreased plasma glucose levels do not appear to be an important factor in the antiaging action of CR (McCarter et al., 2007). However, their similar regulation of several endocrine systems suggests that they may share this mechanism of action. Protein and amino acid restriction may extend lifespan by directly altering the rate or accuracy of translation or protein processing (reviewed in Kaerberlein and Kennedy, 2008; Tavernarakis, 2008), patterns of DNA methylation, glutathione levels (Richie et al., 1994), stress resistance (Miller et al., 2005), or hormesis (Gems and Partridge, 2008).

The rate of translational initiation or elongation appears to be intimately involved in lifespan regulation (Tavernarakis, 2008). Tryptophan and methionine are both essential amino acids, and methionine is the initiating amino acid for translation. Further, protein restriction may limit the amount of essential amino acids available for protein synthesis. Downregulation of protein translation in response to reduced nutrient availability is a highly conserved longevity pathway for invertebrates (Kaerberlein and Kennedy, 2008; Tavernarakis, 2008). Deceleration of protein translation robustly extends the lifespan of *C. elegans* (Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007). Eight of 25 longevity-related genes conserved between yeast and *C. elegans* modulate protein translation [including target of rapamycin (TOR), ribosomal protein S6 kinase (S6K), large subunit ribosomal proteins, and translation initiation factors] (Smith et al., 2008). Thus, a single nutrient-responsive longevity pathway may be conserved downstream of TOR, a nutrient-responsive protein kinase (Kaerberlein and Kennedy, 2008).

Downregulation of protein synthesis may redirect dietary energy away from reproduction and toward repair; thereby reducing protein aggregation and proteotoxicity through decreased demands on the chaperone, repair and degradation pathways (Kaerberlein and Kennedy, 2008). It may also enhance the relative rate of turnover of whole body protein through enhanced proteasomal degradation, autophagy, and (in mitotic tissues) apoptosis (Spindler and Dhahbi, 2007; Cuervo, 2008; Kaerberlein and Kennedy, 2008). CR increases the rate of protein and lipid turnover in mitotic and postmitotic tissues, and cell turnover in mitotically competent tissues (Reviewed in Spindler and Dhahbi, 2007).

2.2.5. Meal frequency and intermittent fasting

The effects of CR are sufficiently robust that meal frequency and composition are not crucially important to its actions. Lifespan is extended by CR in rodents whether food is presented three times per week (Cheney et al., 1983), as a single daily meal (Nelson and Halberg, 1986), or as 2 (Masoro et al., 1995) or 6 meals per day (Nelson and Halberg, 1986). A dietary paradigm sometimes termed “every-other-day feeding” (EOD) or “intermittent fasting” has been reported to extend rodent lifespan since the mid 1940’s (Carlson and Hoelzel, 1946; Goodrick et al., 1983). Although it has been studied as a dietary paradigm distinct from CR, more recent studies measuring food intake have shown that EOD induces mild (~20%) CR (e.g. Caro et al., 2008). Not surprisingly, the effects of EOD feeding are very similar to those of mild CR. They include a lower incidence of diabetes, lower fasting blood glucose and insulin concentrations, and other effects comparable to those of CR (Reviewed in Varady and Hellerstein, 2007).

In humans, reduced meal frequency may have detrimental health effects. A randomized, crossover study in normal weight humans used two 8-week periods during which subjects consumed sufficient calories to maintain their weight as either one or as three meals per day (Stote et al., 2007). Subjects consuming one meal per day had significant increases in blood

pressure and total LDL- and HDL-cholesterol. No significant effects were found on heart rate, body temperature, or most other blood variables. Thus, reduced meal frequency in normal weight humans may produce adverse effects on serum LDL-cholesterol and blood pressure.

2.2.6. Specific nutrients, drugs, and lifespan

In general, the literature describing the testing of specific nutrients and drugs on the lifespan of rodents is confounded (reviewed in Spindler, submitted for publication). Few if any nutrients or drugs have been unequivocally shown to extend the lifespan of healthy rodents. Most such studies do not control, measure, or report caloric consumption or animal weights. Some studies state that weights are unchanged without providing details. Therefore, it is unclear at what age(s) the weights were determined, the number of determinations made, or the statistical significance of the data. Thus, the effects of “voluntary” CR cannot be excluded as a source of the lifespan effects. See Section 2.21 for further discussion of these issues. In addition, most such reports have not confirmed by others. Thus few nutrients have been shown unequivocally to lengthen the lifespan of healthy rodents fed a balanced diet.

There are unconfirmed reports that deprenyl and Dinhlang root extract extend the lifespan of healthy mice (Yen and Knoll, 1992), and that dinitrophenol slightly extends the median and mean lifespan of normal, but relatively short lived mice (Caldeira da Silva et al., 2008). Only 6 others report extended longevity and no change in body weight (with data and data analysis methods shown) as a surrogate measure of food consumption. These 6 treatments are: 2-mercaptoethanol administered orally to male BC3F1 mice (Heidrick et al., 1984); cornstarch vs. sucrose-containing diets fed to Fischer 344 rats (Murtagh-Mark et al., 1995); deprenyl fed to Syrian hamsters (Stoll et al., 1994; Stoll et al., 1997); ginkgo biloba extract administered orally to male Fischer rats (Winter, 1998); green tea polyphenols administered in drinking water to male C57BL/6 mice (Kitani et al., 2007); PBN fed to C57BL/6J male mice (Saito et al., 1998), and piperoxane administered by injection to Fischer 344 rats (Compton et al., 1995). A larger group of studies (Spindler, submitted for publication) report that lifespan is increased without a change in either food consumption and/or body weight. But, either no data are given or the data shown are uninformative. For example, in several studies rodent weights were measured only at the start of the supplement feeding; in others there is no indication of when or how many times during the study that body weights or food consumption were measured, or what statistic was used to analyze the data. Examination of the literature suggests that several of these studies lack sufficient statistical power to detect a 10% change in body weight, and even a small decrease in caloric consumption can lead to lifespan extension in rodents (Compton et al., 1995; Merry, 2002). A supplement that increases water intake or retention, reduces oxygen utilization (metabolic rate), or enhances fat storage could mask changes in calorie consumption. The only way to unequivocally insure that food consumption is unchanged by a treatment is to feed a known amount of food and monitor its consumption.

Notable among the recent studies for which neither body weights nor food consumption are reported are the studies resulting from the NIA Interventions Testing Program (Harrison et al., 2009; Strong et al., 2008; Miller et al., 2007). Reports from these studies include statements to the effect that “body weights were unchanged”, but no data or details are given.

2.3. Evolutionary origin of the health benefits of CR

A study performed using bowl and doily spiders provides a striking example of the evolutionary conservation of the CR

response (Austad, 1989). The mean adult lifespan of the spiders was about 42, 64, or 81 days when they were allowed to consume five, three, or one *Drosophila* per day. As discussed, the longevity and health effects of CR are conserved in species from three Kingdoms and four phyla within Animalia (see Section 2.1).

The most widely accepted theory for the plasticity of lifespan and the similarities of the CR response among species is that it allows individuals to shift energy usage away from growth and reproduction and towards maintenance and stress resistance during times of nutritional stress (Holliday, 1989; Masoro and Austad, 1996; Kirkwood and Austad, 2000). CR delays reproductive maturation; reduces reproductive hormone levels, reproductive behavior, and fertility in adults; and limits the number of offspring (see Sections 2.5 and 2.6, below). When the food supply is restored after a period of CR (as in the Spring, for example) refeeding rapidly returns animals to fertility. The lifespan and other physiological effects of CR also appear to be induced and to dissipate rapidly in response to its initiation and termination (Mair et al., 2003; Dhabhi et al., 2004).

2.4. Longevity mutations and the molecular mechanisms of CR

The search for the mechanisms of action of CR received a major boost when it became clear that downregulation or knockout of gene orthologues involved in the insulin and IGFI signaling systems could produce greater longevity in *C. elegans*, *Drosophila*, and mice (Kenyon, 2005; Russell and Kahn, 2007; Bartke, 2008a). These hormone systems also are downregulated by CR in rodents, monkeys and humans (see Sections 2.1.2, 2.2.1, 2.2.3, 2.2.4, 2.7 and 2.7.2). Ames dwarf mice, which are homozygous for a loss-of-function mutation at the *Prop1* (prophet of Pit1) locus, exhibit 40–70% increases in mean and maximum lifespans compared with their phenotypically normal or non-mutant siblings (Brown-Borg et al., 1996). The Ames mutation ablates anterior pituitary cell lineages, leading to a combined endocrine abnormality that includes low levels of circulating GH, IGFI, thyroid-stimulating hormone, thyroid hormones, and prolactin (Sornson et al., 1996). In Ames and Snell mice, the absence of these hormones postpones many degenerative, age-related changes in physiology, including the development of neoplastic diseases, immune system decline, and collagen cross-linking (Flurkey et al., 2001; Ikeno et al., 2003). Snell dwarf mice, which are homozygous for a loss-of-function mutation in the *Pit1* locus, are deficient in the same anterior pituitary cell types and hormones as the Ames mice, and have an essentially identical longevity and physiological phenotype (Flurkey et al., 2001). Genome wide microarray studies suggest that CR and the Ames mutation affect many of the same genes, pathways, and processes in the liver (Tsuchiya et al., 2004). Ames mice can also respond to CR with further extended lifespan (Bartke et al., 2001). Such data are sometimes mistakenly thought to indicate that CR and the Ames mutation act on distinct signaling and genic pathways. On both theoretical (Gems et al., 2002) and experimental (Tsuchiya et al., 2004) grounds, it is likely that CR and insulin/IGFI signaling use many of the same molecular pathways to extend health and lifespan in mammals.

Among the affected endocrine signaling systems, the IGFI system is likely important for the mammalian CR response. CR strongly downregulates IGFI levels in mammals. Growth hormone receptor knockout (GHRKO) mice have robustly extended lifespan and reduced IGFI levels (Coschigano et al., 2003). Mice homozygous for a loss-of-function mutation in the GH releasing hormone receptor gene, termed *little mice* (Lin et al., 1993), have reduced GH levels and a ~25% increase in lifespan compared to congenic controls (Flurkey et al., 2001). However, IGFR knockout mice do not have a robust longevity phenotype. Only female mice with heterozygous deletion of the IGFI receptor have extended

lifespan (~25% extension) compared to their phenotypically normal siblings (Holzenberger et al., 2003). Males have no increase in lifespan. These results suggest that in addition to the IGF1 signaling pathway, other signaling systems perturbed in the Ames, Snell, and GHRKO mice may also contribute to the lifespan response of CR. Alternatively, while Ames, Snell and GHRKO mice have systemically reduced IGF1 levels, autocrine or paracrine production of IGF1 in other cells and organs, such as brain, may continue and contribute to the health and longevity of these mice. In contrast, abrogation of the local as well as systemic IGF1 action by knockout of the IGFR gene may reduce the overall fitness of the mice by preventing the autocrine and paracrine, as well as the endocrine, actions of IGF1.

Further evidence for the importance of insulin and IGF1 signaling in regulating lifespan is evident in studies of the *Klotho* gene. Mice that overexpress *Klotho* live 31% (males) and 19% (females) longer than congenic, normal controls (Kurosu et al., 2005). An alternative splice product of the *Klotho* gene encodes a circulating protein which blocks autophosphorylation of the occupied IGF1 receptor and promotes dephosphorylation of activated insulin and IGF1 receptors, thereby inhibiting their downstream signaling (Kurosu et al., 2005).

Direct disruption of downstream insulin and IGF1 signaling also increases the lifespan of mice, *Drosophila*, and *C. elegans*. Female (but not male) mice carrying a homozygous knockout of the insulin receptor substrate (IRS)-1 gene live 32% longer than wild type, congenic controls (Selman et al., 2008). These mice have no change in food consumption relative to controls. While these authors found that homozygous and heterozygous knockout of the IRS-2 gene had no effect on lifespan (Selman et al., 2008), others report that knockout mice with reduced IRS-2 levels throughout the body or just in the brain, have an ~18% increase in lifespan (Taguchi et al., 2007). Surprisingly, the long-lived IRS-1 and -2 knockout mice from the studies above have lifelong insulin resistance, which is the opposite of that found with most longevity paradigms. In *Drosophila*, mutational inactivation of the *chico* gene, which encodes a fly homologue of the IRS 1 through 4 genes, reduces insulin/IGF1 signaling, produces a dwarf phenotype, and extends lifespan (~50% extension) (Clancy et al., 2001). Thus, multiple methods of reducing insulin/IGF1 signaling appear able to extend animal lifespan, even when they produce increased insulin resistance, which is normally associated with reduced lifespan. Consistent with the evidence for the importance of insulin and IGF1 signaling in mammalian longevity, reductions in signaling through the TOR pathway using a variety of approaches extend lifespan in yeast (Kaeberlein et al., 2005b; Powers et al., 2006), *C. elegans* (Vellai et al., 2003; Jia et al., 2004), *Drosophila* (Kapahi et al., 2004), and perhaps mice (Harrison et al., 2009) (see below).

Reduced GH receptor signaling itself may be important for the longevity effects of CR independent of its effects on IGF1 levels. GHRKO mice, which have very low levels of circulating IGF1 and insulin, and disrupted GH receptor signaling, have significantly extended lifespans (Coschigano et al., 2000). However, GHRKO mice are unresponsive to either ~15 or ~30% CR (Bonkowski et al., 2006, 2009). As indicated above, Ames dwarf mice are CR responsive, while also having reduced GH, IGF1 and insulin signaling. However, Ames mice also have reduced prolactin, thyrotropin and thyroid hormone levels. Thus, the lack of a CR response in GHRKO mice could be due to the presence of prolactin or thyroid hormone. However, this seems unlikely, since CR leads to a reduction in these hormones. Thus, ablation of GH signaling appears to ablate the mammalian longevity response to CR (Bonkowski et al., 2006). Bartke and his colleagues suggest that this lack of a CR response in GHRKO mice may be related to the failure of CR to increase down stream insulin signaling (sensitivity) in the muscle or liver of these mice (Bonkowski et al., 2009).

2.5. CR and specific signaling systems

A number of transcription factors and transcriptional co-activators have been implicated in the health and longevity effects of CR. Among these, perhaps none have received more attention to date than SirT1 (silent mating type information regulation 2 homolog *S. cerevisiae*), PGC-1 α (peroxisome proliferator activated receptor γ co-activator-1- α), AMPK (AMP-activated protein kinase), and TOR (target of rapamycin). We will consider the evidence for this involvement below.

2.5.1. SirT1

Orthologues of the Sir gene encode a family of NAD-dependent protein deacetylases which are termed “sirtuins” (Imai et al., 2000; Landry et al., 2000). Under some conditions, sirtuins appear to be required for lifespan extension by CR in yeast (Lin et al., 2000), *Caenorhabditis elegans* (Wang and Tissenbaum, 2006), and *Drosophila* (Rogina and Helfand, 2004). Further, an additional copy of the orthologous gene can increase lifespan of yeast (Kaeberlein et al., 1999), *C. elegans* (Tissenbaum and Guarente, 2001), and *Drosophila* (Rogina and Helfand, 2004) by 18–50%. However, the relationship between lifespan and these sirtuin orthologues is complex, and still poorly understood, even in yeast. For example, at 0.5% glucose, which is defined as CR in some yeast studies, lifespan extension appears to require Sir2 (the yeast orthologue of SirT1) and nicotinamide adenine dinucleotide (NAD) (Lin et al., 2000). However, at 0.05% glucose, replicative lifespan may be extended by a Sir2-independent mechanism (Kaeberlein et al., 2004; Kaeberlein et al., 2005a). Further, CR-related activation of Sir2 in *S. cerevisiae* has been thought to increase replicative lifespan by silencing ribosomal DNA, thereby suppressing the generation of extrachromosomal rDNA circles by recombination during DNA replication. Accumulation of these circles reduces replicative lifespan in yeast. However, two recent studies have challenged these assumptions (Riesen and Morgan, 2009; Smith et al., 2009). In both studies, CR-mediated repression of rDNA recombination (accomplished using 0.05 or 0.5% glucose) was shown to be independent of the silencing of rDNA and other DNA loci by Sir2. Thus, the role of Sir2 and gene silencing in determining the replicative lifespan of yeast remains to be clarified.

The mammalian SirT1 sirtuin gene is the closest human ortholog to the yeast Sir2 gene. It encodes a sirtuin that deacetylates transcription factors and coactivators with key roles in metabolism and stress resistance, including p53 (Vaziri et al., 2001), FOXO (Brunet et al., 2004; Motta et al., 2004), PGC-1 α (Rodgers et al., 2005), and NF- κ B (nuclear factor- κ B) (Yeung et al., 2004). For example, PGC-1 α is activated when it is deacetylated by SirT1. The potential role of SirT1 in aging and CR in mammals is even less defined than that of Sir2 in yeast. CR does not extend the lifespan of SirT1 null mice (Chen et al., 2005). However, the meaning of this observation is unclear. SirT1-null mice have multiple, severe abnormalities (McBurney et al., 2003). They are small, with developmental defects of the retina and heart. On an inbred background, they usually do not survive postnatally (Cheng et al., 2003). On an outbred genetic background, most do survive to adulthood (McBurney et al., 2003). But, these mice are small and sterile, with craniofacial abnormalities, and an eyelid inflammatory condition. They are hypermetabolic, utilize ingested food inefficiently, have inefficient liver mitochondria, and have elevated rates of lipid oxidation (Boily et al., 2008). The absence of lifespan extension in these mice has been interpreted as evidence that SirT1 is mechanistically required for the CR response (Haigis and Guarente, 2006). However, it is equally possible that the molecular pathways engaged by CR are functional in SirT1-null mice, but are already fully induced by the stress caused by the gene knockout (Schumacher et al., 2008). For example, Schumacher et al., showed

that while mice carrying knockins of human DNA repair-deficient progeroid syndrome genes die prematurely, they strongly induce a genome-wide pattern of gene-expression which is highly homologous to the gene expression patterns found in long-lived CR mice, Ames and Snell dwarf mice, CR-Ames dwarf mice, and growth hormone receptor knockout mice (Schumacher et al., 2008). The endocrine, metabolic, and gene expression changes induced by these longevity manipulations appear to induce a “survival”-related genetic program which is also induced in the progeroid mice. The SirT1 knockout mice may also induce this survival pathway, thereby negating a further CR response.

2.5.2. PGC-1 α

The mitochondrial capacity for oxidative phosphorylation (ATP production) declines with advancing age in human skeletal muscle (Short et al., 2005). This decrease is accompanied by an age-related decline in muscle mitochondrial number and function (Short et al., 2005). Because mitochondria are key participants in glucose and lipid catabolism, mitochondrial dysfunction reduces muscle carbohydrate and lipid uptake and catabolism, thereby increasing systemic dyslipidemia and hyperglycemia (Petersen et al., 2007; Kim et al., 2008). These changes appear to be integral to the age-related development of insulin resistance and metabolic syndrome (Eckel et al., 2005; Petersen et al., 2007; Kim et al., 2008). Metabolic syndrome aggravates age-related inflammation, hypertension, and cardiovascular disease (Kim et al., 2008).

PGC-1 α is a transcriptional co-activator and a central regulator of mitochondrial biogenesis, oxidative phosphorylation, hepatic gluconeogenesis, fatty acid oxidation, and muscle fiber type (Puigserver et al., 1998; Lopez-Lluch et al., 2008; Scarpulla, 2008; Ventura-Clapier et al., 2008; Canto and Auwerx, 2009). PGC-1 α interacts with and integrates the activities of a diverse set of key transcription factors, including PPAR α , NRF-1, NRF-2, ERR α , and mtTFA (Puigserver et al., 1998, 1999; Schreiber et al., 2004; Gleyzer et al., 2005). Through such interactions, an increase in PGC-1 α level or activity upregulates mitochondrial biogenesis, fatty acid oxidation, and muscle fiber type switching to type I fibers, which have a higher mitochondrial content and oxidative rate than type IIb fibers (Puigserver et al., 1998; Wu et al., 1999; Vega et al., 2000; Canto and Auwerx, 2009). The induction of PGC-1 α expression may also have a salutary effects on the survival of some cell types. For example, lentiviral overexpression of PGC-1 α in the striatum protects neurons from atrophy in the R6/2 mouse model of Huntington's disease (Cui et al., 2006).

The activity of the PGC-1 α gene is regulated by CREB (cAMP response element-binding protein) coactivator TORC (transducer of regulated cAMP response element-binding protein) family members (Wu et al., 2006). TORC 1, 2, and 3 strongly induce PGC-1 α expression in skeletal muscle cells (Wu et al., 2006). This leads to upregulation of the mitochondrial respiratory chain and tricarboxylic acid cycle genes, increasing cellular respiration and fatty acid oxidation (Wu et al., 2006).

CR maintains PGC-1 α levels in muscle during aging, thereby preserving mitochondrial function and biogenesis (Baker et al., 2006; Hepple et al., 2006). Maintenance of skeletal muscle PGC-1 α levels may involve signals originating in the hypothalamus. CR and the longevity-related dwarf mutations enhance fatty acid β -oxidation and inhibit fatty acid biosynthesis (Tsuchiya et al., 2004; Spindler and Dhahbi, 2007). Pharmacologic inhibition of fatty acid synthase has been shown to increase the number of mitochondria in white and red (soleus) skeletal muscle by increasing the level of malonyl-CoA (the substrate of fatty acid synthase) in the hypothalamus (Cha et al., 2006). This malonyl-CoA accumulation increases signaling through the sympathetic nervous system to the skeletal muscle. In the muscle, this signaling induces the expression of the β -adrenergic signaling molecules norepinephr-

ine, β 3-adrenergic receptor, and cAMP, thereby inducing PGC-1 α and estrogen receptor-related receptor- α expression, and mitochondrial biogenesis (Cha et al., 2006, and references therein). Exercise induces mitochondrial biogenesis in muscle through a signaling cascade beginning with elevated intracellular free calcium and culminating in enhanced PGC-1 α activity (Wu et al., 2002).

2.5.3. AMPK

Like PGC-1 α , reduced skeletal and heart muscle AMPK activity may have a key role in the decline in mitochondrial biogenesis, insulin sensitivity, and lipid metabolism in older animals and humans (Qiang et al., 2007; Reznick et al., 2007). As reviewed above, age-related reductions in mitochondrial number and function contribute to dysregulated intracellular lipid metabolism, which leads to increased insulin resistance in older animals and humans (Petersen et al., 2003). In skeletal muscle cells, increased AMPK activity enhances fatty-acid oxidation by directly phosphorylating and activating PGC-1 α and acetyl-CoA carboxylase 2 (ACC2) (Merrill et al., 1997; Winder et al., 2006; Jager et al., 2007).

Enhanced AMPK signaling is capable of extending the lifespan of *C. elegans*, *Drosophila*, and yeast (Tschape et al., 2002; Apfeld et al., 2004; Harkness et al., 2004). However, its involvement in the lifespan effects of CR in these organisms remains uncertain. For example, the *C. elegans* ortholog of the AMPK α subunit, *aak-2*, is not required for the lifespan effects of *eat-2* mutants, which are thought to induce CR (Curtis et al., 2006).

The mammalian AMPK is a heterotrimer of a catalytic α subunit and regulatory β and γ subunits (Hardie et al., 2003; Carling, 2004). There are multiple genes for each of these subunits (α 1, α 2, β 1, β 2, γ 1, γ 2, γ 3). The use of alternative promoters and splice sites for these subunits further increases the structural complexity of the kinase. In mammals, AMPK is allosterically activated by AMP, and is therefore responsive to the intracellular ratio of AMP to ATP (Hardie et al., 2003). In mammals, energy deficits result in elevated intracellular AMP, which binds to AMPK and increases its interaction with LKB1, which is itself a kinase. This interaction leads to phosphorylation and activation of AMPK. This activation initiates a signaling cascade which stimulates glucose uptake and fatty acid oxidation, and downregulates energy-requiring processes such as protein synthesis in cultured skeletal muscle cells (Alessi et al., 2006). The phosphorylation and activation of TSC2 (tuberous sclerosis 2 protein) by activated AMPK also can protect cultured HEK293 (human embryonic kidney 293) cells (an adenovirus-DNA transformed cell line) from energy deprivation-induced apoptosis, suggesting that AMPK also can enhance cell survival (Inoki et al., 2003).

AMPK also responds to, and regulates, food intake and systemic energy expenditure by responding to hormonal signals in the central nervous system and peripheral tissues (Kahn et al., 2005). AMPK activity is induced by the systemically active cytokines leptin and adiponectin, which are also termed adipokines, since they are secreted by adipose. The AMPK pathway is required for the metabolic- and insulin-sensitizing actions of these adipokines. Leptin selectively stimulates threonine 172 (Thr-172)-phosphorylation of the AMPK α 2-catalytic subunit in skeletal muscle, activating it (Minokoshi et al., 2002). Stimulation involves an early, direct effect of leptin on muscle, as well as an indirect, more sustained activation involving the action of the hypothalamic-sympathetic nervous system on α -adrenergic receptors, leading to fatty acid oxidation in muscle (Minokoshi et al., 2002). Adiponectin stimulates AMPK phosphorylation and activity in muscle and liver in vivo and in vitro (Yamauchi et al., 2002). In muscle, AMPK activation is required for adiponectin responsive stimulation of fatty acid oxidation and glucose transport (Yamauchi et al., 2002). In liver, AMPK activation is required for adiponectin-responsive

inhibition of PEPCK, glucose-6-phosphatase, and hepatic glucose production (Yamauchi et al., 2002). AMPK also regulates food intake by responding to hormonal and nutrient signaling in the hypothalamus (reviewed by Kahn et al., 2005).

Because mammalian AMPK is responsive to cytokines, fasting is a potent inducer of its activity. Just 6 h of food deprivation in rats increases the level of the Thr-172-phosphorylated, AMPK α -subunit in gastrocnemius muscle by approximately 4-fold (de Lange et al., 2006). Fasting also increases the level of nuclear PGC-1 α and PPAR γ (de Lange et al., 2006). AMPK is also activated by exercise (Zhou et al., 2001).

However, CR does not produce effects on AMPK activity similar to those produced by fasting. In mice, LTCR does not change AMPK α 1 or - α 2 activities measured in cell-free extracts of heart or skeletal muscle (Gonzalez et al., 2004b). Consistent with this result, LTCR has little effect on the level of Thr-172-phosphorylated AMPK α in the quadriceps femoris muscle of Wistar rats (To et al., 2007). CR has been reported to increase AMPK activity measured in cell-free liver extracts by approximately 20% (Gonzalez et al., 2004b). However, others reported that CR decreases levels of Thr-172-phosphorylated AMPK α in the liver of Wistar rats (To et al., 2007). STCR is reported to increase myocardial levels of Thr172-phosphorylated AMPK α in both young and old male Fischer 344 rats (Shinmura et al., 2005). Thus, CR appears to have little or no effect on AMPK activity in skeletal muscle or liver, but may increase its activity in rat heart.

AMPK is activated in mammals by metformin treatment and by exercise (Zhou et al., 2001; Hadad et al., 2008). Metformin is a small-molecule biguanide which increases peripheral insulin sensitivity (Widen et al., 1992), increases glucose uptake in skeletal muscle (Galuska et al., 1991; Hundal et al., 1992), decreases hepatic gluconeogenesis (Johnson et al., 1993; Hundal et al., 2000), and inhibits the activity of complex I of the mitochondrial respiratory chain (El Mir et al., 2000; Owen et al., 2000; Detaille et al., 2002; Zou et al., 2004). Metformin therapy reduces blood glucose levels in type 2 diabetics, and inhibits the development of metabolic syndrome (Orchard et al., 2005). Metabolic syndrome is associated with increased cardiovascular- and diabetes-associated morbidity and mortality. At least a part of the physiological pathways engaged by CR also appear to be targeted by metformin. Eight weeks of oral metformin treatment of old mice reproduces approximately 75% of the genome-wide, gene expression changes produced by either LTCR or 8 weeks of CR (Dhahbi et al., 2005).

Metformin usage may be associated with a reduced risk of cancer in humans and animals. A small-scale, population-based, case-control study found that diabetics who take metformin are 23% less likely to get cancer compared with those taking sulfonylurea (Evans et al., 2005). The risk was reduced by 40% for people who took the drug for a longer time. A second population based cohort study found that new users of metformin had a significantly reduced risk of cancer mortality relative to those using sulfonylureas or exogenously administered insulin (Bowker et al., 2006). However, these studies do not distinguish the potential pro-cancer effects of elevated insulin exposure in the sulfonylurea and insulin treated groups from the putative protective effects of metformin on the development of cancer.

Chronic administration of metformin to female, transgenic HER-2/neu mice, which are a breast-cancer model, increased their mean and maximum life span by 8% and 13%, respectively (Anisimov et al., 2005a,b). However, metformin treatment also decreased their food consumption at 4 and 6 mo of age, suggesting that “voluntary CR” may be the source of this anticancer response. SHR mice, a normal but short-lived strain, experienced nearly a 38% and a 21% increase in mean and maximum lifespan when chronically treated with metformin (Anisimov et al., 2008).

However, metformin also decreased food consumption in these studies, suggesting CR may be responsible for at least a part of the lifespan effects. In another study, a low dose of metformin increased the survival of male R6/2 mice (a short-lived, transgenic model of Huntington’s disease) by approximately 20% (Ma et al., 2007). However, food consumption was not monitored and the weights of the animals decreased rapidly with age. Phenformin, a biguanide which is structurally and functionally closely related to metformin, extended the lifespan of C3H mice by ~23% and reduced tumor incidence by 80% (Dilman and Anisimov, 1980; Anisimov et al., 2003). Again, food consumption was not reported. Recent studies have shown that metformin inhibits breast cancer cell growth, colony formation, and induces cell cycle arrest in vitro (Alimova et al., 2009). These results are consistent with the idea that activation of AMPK by metformin may reduce cancer-related mortality. However, it remains to be shown that either metformin or phenformin can extend lifespan independently of CR (Reviewed in Spindler, submitted for publication).

2.5.4. TOR

TOR is a nutrient-responsive protein kinase that integrates signals from growth factors, hormones, nutrients, and cellular energy to regulate (depending on species and cell type) cell growth, proliferation, survival, protein translation, nutrient transport, endocytosis, glycogen accumulation and autophagy (Wullschleger et al., 2006; Arsham and Neufeld, 2006; Heitman et al., 1991). As described above, disruption of TOR signaling can extend the lifespan of yeast, *C. elegans*, *Drosophila*, and perhaps mice (Harrison et al., 2009 and references given above; and see Section 2.2.1 below). The mammalian TOR protein, mTOR, a conserved serine/threonine kinase, is present as two multisubunit protein complexes, termed mTORC1 (mTOR complex 1) and mTORC2. mTORC1, which is inhibited by the macrocyclic lactone antibiotic, rapamycin (sirolimus), contains three subunits in addition to mTOR, Raptor (rapamycin-sensitive adaptor protein of mTOR), mLST8 (a.k.a. G β L), an ortholog of yeast LST8, which binds specifically to the protein kinase domain of mTOR (reviewed in Bhaskar and Hay, 2007) and proline-rich Akt substrate of 40 kilodaltons (PRAS40). mTORC1 also binds the small G-protein Rheb, which stimulates mTOR kinase activity (Long et al., 2005a).

In the mouse lifespan study cited above, the activity of mTORC1 was inhibited by feeding mice microencapsulated rapamycin beginning late in life (Harrison et al., 2009). Lifespan extension might not be predicted based on the whole animal effects of this drug and its more bioavailable analogues, such as temsirolimus and everolimus (sometimes termed rapalogues). Mice (Cunningham et al., 2007; Bussiere et al., 2006) or gerbils (Fraenkel et al., 2008) treated with rapamycin or rapalogues become diabetic and hyperlipidemic. Treated mice develop hyperglycaemia, glucose intolerance, higher levels of free fatty acids in skeletal muscle, hypertriglyceridemia and hypercholesterolemia (Cunningham et al., 2007). Rapamycin treatment causes smaller pancreatic islets, a decrease in glucose-stimulated insulin synthesis and secretion, and a 90% reduction in serum insulin levels (Fraenkel et al., 2008). Rapamycin and its rapalogues are also immunosuppressants (Kahan, 2008).

Rapamycin feeding late in life most likely extended mouse lifespan through its anticancer effects (Brachmann et al., 2009; Liu et al., 2009; Kahan, 2008; Wullschleger et al., 2006; Bjornsti and Houghton, 2004). As with most mouse strains (e.g. Dhahbi et al., 2004), a majority of the strain of mice used for the rapamycin lifespan studies die from cancer (Miller and Chrisp, 2002). Rapamycin and a number of rapalogues have been used as anti-cancer drugs (Liu et al., 2009). Rapamycin or rapalogues inhibit the in vitro or in vivo proliferation of many tumor cell types, including those derived from childhood rhabdomyosarcomas tested in vitro

(Dilling et al., 1994), human medulloblastoma and glioma tumors tested as xerographs in nude mice (Houchens et al., 1983), and panels of tumor cells from the Pediatric Preclinical Testing Program tested in vitro and in vivo as xenografts in nude mice (Houghton et al., 2008).

The activation of mTORC1 by growth factors such as insulin and IGF1 results from tyrosine phosphorylation and activation of downstream scaffolding adaptors such as insulin receptor substrate 1 (IRS1). They bind and activate phosphatidylinositol 3 (PIP3) kinase, thereby increasing production of PIP3 (Manning and Cantley, 2007). PIP3 is bound by the serine/threonine kinase, Akt (a.k.a. protein kinase B; PKB) and phosphoinositide-dependent kinase-1 (PDK1). PDK1 phosphorylates a key tyrosine on Akt, which phosphorylates a tyrosine on TSC2. This phosphorylation inactivates the TSC1/TSC2 complex, which blocks its ability to hydrolyze the GTP bound to Rheb (Huang and Manning, 2008). This allows Rheb-GTP to accumulate. Rheb-GTP binds to, and activates the serine/threonine kinase activity of mTORC1 (Huang and Manning, 2008). Akt also activates mTORC1 by phosphorylating PRAS40, which relieves the inhibition of mTORC1 activity by bound PRAS40.

mTORC1 activation up-regulates protein synthesis (a major impetus for cell growth), largely through mTORC1 binding to eukaryotic translation initiation factor 3 (eIF3), where it phosphorylates the translational regulators ribosomal protein S6 kinases 1 and 2 (S6K1 and S6K2) (Wang et al., 2005; Hay and Sonenberg, 2004), and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) (Holz et al., 2005). In addition to phosphorylating TSC2, activated PDK1 also directly phosphorylates and activates the S6Ks. Phosphorylated S6K1 stimulates translation by phosphorylating Ser366 of eukaryotic elongation factor 2 (eEF2) kinase (Wang et al., 2001). eEF2 kinase is also phosphorylated on Ser359 by cdc2/cyclin B, strongly inhibiting its activity (Knebel et al., 2001). The inactivated kinase can no longer phosphorylate and inactivate eEF2, which is free to efficiently mediate the translocation step of peptide-chain elongation (Merrick and Nyborg, 2000).

As mentioned above mTORC1 also phosphorylates 4EBP1, which controls the activity of eukaryotic initiation factor eIF4E. eIF4E binds the 5'-cap of eukaryotic mRNA, facilitating ribosome recruitment. 4EBP1 binds eIF4E, inhibiting its binding to the 5'-cap, thereby inhibiting translation. mTORC1 phosphorylation of 4EBP1 relieves this inhibition, activating translation initiation, and stimulating the overall rate of protein synthesis.

Thus, mTORC1 activation strongly stimulates protein synthesis, which leads to cell growth and division. But, active mTORC1 and S6Ks also phosphorylate serine residues on IRS1, which targets IRS1 for degradation (reviewed in Manning and Cantley, 2007). This is one of multiple negative feedback loops which finally attenuate the mTORC1-related signaling cascade (Reviewed in Polak and Hall, 2009).

In yeast cells, drosophila embryos and cells in culture, the activation of TOR inhibits macroautophagy, while amino acid or glucose starvation, or withdrawal of insulin or other growth factors inhibits TOR activity and stimulates macroautophagy (Lum et al., 2005; Kamada et al., 2004; Edinger and Thompson, 2007). Inhibition of TOR by amino acid deprivation or rapamycin treatment downregulates anabolic and cell growth-related processes such as protein synthesis and ribosome biogenesis, as described above, and up-regulates catabolic and growth inhibitory processes such as macroautophagy. Starving cells for amino acids reduces Rheb binding to mTORC1, reducing mTORC1 activity (Long et al., 2005b). The mechanism for these effects remain unknown. The extent to which nutrient sufficiency or insufficiency regulates macroautophagy through mTORC1 in mammals also unclear at this time. The macroautophagy studies described above were per-

formed with yeast, *Drosophila* embryos, or (in a few cases) cultured mammalian cells.

2.6. Reproductive effects of CR in rodents and flies

Studies of *Drosophila*, rats, and mice often find that females outlive males. This is also true when these species are subjected to CR (e.g. Cheney et al., 1983; Sheldon et al., 1995; Turturro et al., 1999; Magwere et al., 2004; Partridge et al., 2005). For example, female medflies live significantly longer than male flies at all food levels (Davies et al., 2005). While this relationship holds for many strains of mice, it is not universally true of all strains (Blackwell et al., 1995). The molecular basis for these sexual dimorphisms is not known. But in mice, they may result from the differential effects of the sex steroids on cancer cell growth.

CR and mutations that reduce IGF1 signaling attenuate, delay, or abolish fertility and fecundity in *Drosophila*, *C. elegans*, and female rats and mice (Klass, 1977; Holehan and Merry, 1985b; Weindruch and Walford, 1988; Chippindale et al., 1993; Kenyon et al., 1993; Chapman and Partridge, 1996; Gems et al., 1998; Tissenbaum and Ruvkun, 1998; Bruning et al., 2000; Burks et al., 2000; Clancy et al., 2001; Tatar et al., 2001; Chandrashekar and Bartke, 2003; Giannakou et al., 2004). CR begun after weaning delays sexual maturation and the loss of estrous cyclicity in aging female mice, perhaps by retarding the rate of follicular depletion (Merry and Holehan, 1979; Holehan and Merry, 1985b; Nelson et al., 1985; Gonzales et al., 2004). Oocyte numbers are higher in CR females when compared to age-matched ad libitum controls (Lintern-Moore and Everitt, 1978; Nelson et al., 1985). Female mice and rats maintained on CR from an early age and returned to ad libitum feeding later in life are restored to reproductive cycling and reproductive competence at chronological ages where ad libitum animals have long ceased these activities (Osborne et al., 1917; Ball et al., 1947; Visscher et al., 1952; Holehan and Merry, 1985a; Nelson et al., 1985).

CR begun later in life causes female rats and mice to cease reproductive cycling and reproduction (Ball et al., 1947; Visscher et al., 1952; Nelson et al., 1985). Even moderate levels of CR initiated in rodents during adulthood can sustain the function of the female reproductive axis into advanced chronological age (Selesniemi et al., 2008). These findings suggest that the beneficial effects of CR on female reproductive function are at least partly mediated via maintenance of the ovarian follicular reserve. However, CR affects the secretion patterns of hormones produced by the hypothalamus and pituitary, which participate in the control of ovarian function (Martin et al., 2008). Hence, the effects of CR on reproductive performance probably involve the entire hypothalamic-pituitary-gonadal axis.

LTCR reduces male reproductive activity. Male CFY Sprague-Dawley rats subjected to LTCR sire significantly fewer litters at most ages (Merry and Holehan, 1981). They also have a delayed and reduced pubertal peak of serum testosterone, delayed puberty, and reduced testosterone levels (Merry and Holehan, 1981). Males subjected to 25% CR were less able to attract females, and those on 50% CR were both less able to attract and to sexually arouse females (Govic et al., 2008). Thirty days of CR in adult male Wistar rats reduces serum and testicular testosterone concentrations (Santos et al., 2004). Only one study reported that LTCR increases serum testosterone levels at all ages in Lobund-Wistar rats (Snyder et al., 1988). Sex-related differences have been reported in the energy balance of young CR Wistar rats. CR females deactivate facultative thermogenesis to a greater degree than males, conserving metabolically active organ mass and decreasing adipose depots to a greater extent than restricted males. This ability likely has survival advantages for females when food is limiting, due to their reproductive roles (Valle et al., 2005).

Together these results suggest that CR delays and suppresses sexual maturation, reproductive hormone levels, and reproduction in both male and female rats. It also extends reproductive lifespan, and delays reproductive senescence when older female rodents are shifted from a CR regimen to ad libitum feeding.

The response to CR also is sexually dimorphic in *Drosophila* (Magwere et al., 2004). The peak extension of longevity occurs at lower food concentrations for males than for females, probably because females devote energy to egg-laying (Magwere et al., 2004). In addition, CR increases the longevity of females 60%, while for males the increase is only 30%. Male and female *Drosophila* also exhibit sex-specific differences in the sensitivity of their lifespan to insulin/IGFI signaling, nutrient/energy demand, and allocation and utilization of energy (Clancy et al., 2001, 2002).

Thus, both the physiology and hormonal responsiveness of males and females appear to lead to sex specific differences in their responsiveness to CR across widely divergent species.

2.7. Reproductive effects of CR in humans

In humans, fasting and extremely low calorie diets are well-known to suppress the pituitary–gonadal axis, and free testosterone levels in males (Veldhuis et al., 1993, and references therein). However, neither the Biosphere nor the CALERIE studies reported a decrease in sex steroid levels in adult humans subjected to a CR diet for up to two years (Walford et al., 2002; Holloszy and Fontana, 2007; Redman and Ravussin, 2009). The reasons for the dissimilarities between these results and those found with CR rodents and fasting humans are unclear. The obvious possibilities are that the Biosphere and CALERIE diets were not of sufficient severity or length to produce suppressive effects on sex steroid levels. However, this seems unlikely based on the decrease in BMI obtained and the length of the studies. Thus, humans appear to be less susceptible than rodents to suppression of the hypothalamic-pituitary-gonadal axis by CR. Differences in the life-histories of primates and rodents may have selected for less reproductive suppression in humans in response to nutrient deprivation. This may reflect the relatively longer lifespan and gestation time, and the fewer number of offspring per pregnancy in humans.

2.8. Anticancer effects of CR in rodents

There is compelling evidence that LTCR, STCR, and many of the longevity-enhancing mutations in mice can dramatically delay tumor-associated mortality (Massaro et al., 2004; Kennedy et al., 2003; Massaro et al., 2007; Spindler and Dhahbi, 2007). The anticancer effects of CR in rodents were recognized a century ago (Moreschi, 1909), long before its effects on longevity were published (McCay et al., 1935; reviewed in Weindruch and Walford, 1988). A widely accepted model of carcinogenesis proposes that it involves 3 stages: *initiation*, an initial mutation leading to cell division; *promotion*, accrual of additional mutations in cell growth or proliferation-related genes accompanied by clonal expansion; and *progression*, significant clonal expansion and genetic damage resulting in a gain of tumor mass through increased rates of cell proliferation and/or reduced rates of apoptosis (Hursting et al., 2003). The relative rates of proliferation and apoptosis during the promotion and progression stages of tumorigenesis are the major determinants of the rates of tumor onset and growth (Grasl-Kraupp et al., 1997; Hursting et al., 2003; Patel et al., 2004). In model tumors, LTCR can reduce carcinogenesis at each stage of the multistage model (Hursting et al., 2003; Patel et al., 2004). There is only one report of the effects of CR on the growth rate of spontaneously initiated tumors (Spindler, 2005). The results of this study suggest that CR reduces the growth rate of spontaneous tumors by increasing the rate of tumor cell death (S.R.

Spindler, Y. Higami and I. Shimokawa, unpublished results; and Dhahbi et al., 2004).

LTCR increases the age of onset of tumors in rodents (Hursting et al., 2003). It also decreases the rate of growth and number of metastases produced by many model tumors, including hepatomas, mammary carcinomas, and prostatic tumors (Hursting et al., 2003). CR suppresses carcinogenesis induced by several classes of chemicals, inhibits several forms of radiation-induced cancer, and inhibits neoplasia in early-tumor-onset knockout and transgenic mouse models (Patel et al., 2004). Even when begun late in life, CR decreases spontaneous tumor-associated mortality by 3.1-fold within 8 weeks, and extends both the mean and maximum lifespan of mice (Dhahbi et al., 2004; Spindler, 2005).

There are conflicting reports about whether CR can mitigate prostate carcinogenesis in rats (Boileau et al., 2003; McCormick et al., 2007). The induction protocols used for these studies are complex, and it is unclear how well they recapitulate spontaneous prostatic carcinogenesis.

2.8.1. Apoptosis and the anticancer effects of CR

Fasting, STCR and LTCR reduce cellular proliferation and increase apoptosis in a wide variety of organs and tissues, including liver (Merry and Holehan, 1985; Grasl-Kraupp et al., 1994; James and Muskhelishvili, 1994; Muskhelishvili et al., 1996; Hikita et al., 1999; Higami et al., 2000), bladder (Lok et al., 1988; Dunn et al., 1997), skin (Merry and Holehan, 1985; Lok et al., 1988), kidney (Merry and Holehan, 1985), heart (Merry and Holehan, 1985), mammary gland, esophagus, jejunum (Lok et al., 1988), and colorectum (Lok et al., 1988; Premoselli et al., 1997). Preneoplastic and neoplastic cells are more sensitive to apoptotic cell death than normal cells, and they are selectively eliminated by CR. For example, in mice, 3 months of 40% food restriction significantly reduced the proportion of the liver occupied by preneoplastic foci (Grasl-Kraupp et al., 1994). The increased rate of apoptosis is significant because, as stated above, the relative rates of proliferation and apoptosis are the major determinants of the rates of tumor onset and growth (Hursting et al., 2003; Patel et al., 2004).

The rate of apoptosis in the liver of mice is 3-times higher in hepatocytes from CR mice at all ages (James and Muskhelishvili, 1994; Muskhelishvili et al., 1995; James et al., 1998). 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, 1–3 months of food restriction can significantly increase the latency and reduce the incidence of spontaneous cancer over the entire lifespan of a mouse (Klebanov, 2007). Just one week of CR induces apoptosis in preneoplastic liver cells of old mice (Muskhelishvili et al., 1996). Forty-percent food restriction for 3 months eliminates 20–30% of liver cells through apoptosis, and reduces the number and volume of chemically induced preneoplastic foci by 85% (Grasl-Kraupp et al., 1994). 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 (Luan et al., 1995; Holt et al., 1998).

Thus, LTCR, STCR, and disruption of IGFI signaling appear to exert at least a part of their anticarcinogenic activity by preferentially inducing apoptosis in tumors and preneoplastic foci.

2.8.2. IGFI and insulin receptor signaling, and cancer

Oncogenesis may involve activation of oncogenes, overexpression of growth factors, inappropriate growth factor signaling, and/or inactivation of tumor suppressor genes (Alexia et al., 2004, 2006; Pandini et al., 2004; Boissan et al., 2005; Huether et al.,

2005b,a; Hopfner et al., 2006). LTCR, STCR, and many of the longevity-enhancing mutations in mice dramatically reduce insulin and IGFI serum levels and post-receptor signaling, delay tumor-associated mortality, and increase apoptosis in mitotic tissues (Dhahbi et al., 2001; Dominici et al., 2002; Kennedy et al., 2003; Koubova and Guarente, 2003; Bartke, 2005, 2006). IGFR plays a major role in mitogenesis, transformation, and protection from apoptosis. In many tumors, the level of IGFR expression correlates inversely with survival, and directly with disease stage, metastases and tumor de-differentiation (Scharf and Braulke, 2003; Sedlaczek et al., 2003; Yao et al., 2003). Reduction of IGFR signaling in mice by mutation, genetic manipulation, or Klotho overexpression enhances the rate of tumor cell apoptosis, reduces the number and the volume of preneoplastic lesions, delays tumor-associated mortality, and extends lifespan (Bartke and Brown-Borg, 2004; Bartke, 2006). A part of the antineoplastic effects of CR is probably a direct result of reduced IGFR signaling (Dunn et al., 1997; Hopfner et al., 2006). Cells that do not express IGFR are resistant to transformation by any means (Baserga et al., 2003).

LTCR also increases insulin sensitivity (glucose uptake and utilization) about 4-fold in rats and mice, and reduces blood insulin levels to about half those found in control rats and mice (Masoro et al., 1992; Dhahbi et al., 2001). Lower insulin levels also may be important in the anticancer effects of CR. According to the classical view, the IR predominantly mediates anabolic effects, while the IGFR predominantly mediates antiapoptotic, mitogenic, and transforming effects (Baserga et al., 2003; Biddinger and Kahn, 2006). Insulin receptor A (IRA), a splice variant derived from the same primary transcript as IRB (the isoform which mediates insulin-responsive glucose uptake, metabolism, protein synthesis, and cell growth in muscle and adipose), has a mitogenic and transforming role in oncogenesis. Both the IRA and IRB are expressed in most insulin responsive tissues (Kosaki and Webster, 1993; Kosaki and Webster, 1993). Insulin, IGFI and IGFI are high affinity activators of IRA, and activation can promote growth and protect malignant cells from apoptosis (Kalli et al., 2002; Sciacca et al., 2002; Vella et al., 2002). Binding of IGFI to IRA induces a different, but partially overlapping, gene expression profile than insulin binding (Pandini et al., 2003; Pandini et al., 2004). In cells expressing IRA, IGFI stimulates the Shc/ERK branch of the insulin/growth factor signaling pathway, inducing mitogenesis and migration more potently than insulin (Frasca et al., 1999). In contrast, insulin is more potent than IGFI in stimulating signaling through the IRS/AKT pathway, leading to enhanced glucose metabolism, protein synthesis, and cell growth (Frasca et al., 1999).

Thus, the CR mediated reductions in serum levels of IGFI and insulin may be keys to its anticancer activity. However, the potential effects of CR on other growth factors and their receptors are unexplored. Autocrine and paracrine stimulators of tumor cell growth and division may be altered by CR. Thus, we still do not know whether the effects of CR on insulin and IGFI levels are the primary mechanisms for reduced carcinogenesis in mammals.

2.8.3. Can life history account for the anticancer effects of CR?

Relatively few cancers arise from postmitotic cells (Wright and Deshmukh, 2006). Some or all cancers probably arise from mitotically competent cells, perhaps from stem cells (Chiang and Massague, 2008). Cell division is required to genetically fix oncogenic mutations in the genome. Selection can act only on pre-reproductive or reproductively active members of a population, and in many species few animals live long enough to die of cancer in the wild (e.g. Phelan and Austad, 1989). Highly effective molecular mechanisms have evolved to suppress the development of cancer until after reproduction ceases (Sharpless, 2005). These include mechanisms for high fidelity DNA replication and repair, and tumor suppressor genes such as p53, Rb, p16^{INK4a} and

ARF (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006).

Thus, the anticancer effects of CR may have co-evolved with another phenotype, which is subject to natural selection. We have argued that this trait is the metabolic role of tissues as reservoirs of metabolic energy (Spindler and Dhahbi, 2007). CR animals alternate between balanced waves of turnover and resynthesis (Spindler and Dhahbi, 2007). The minimal energy stores in CR animals demand that the immediate postprandial period for them is characterized by intense biosynthetic activity, including cell division, under the powerfully anabolic influence of insulin (Spindler and Dhahbi, 2007). During the later postprandial period, as insulin levels decline and glucagon and other catabolic hormones rise, high relative rates of protein, lipid, organelle and cellular turnover take place (Spindler and Dhahbi, 2007). Thus, CR drives repeated cycles of cell and tissue turnover in mitotically competent tissues and tissue turnover and repair in postmitotic tissues. These effects seem to be major contributors to the multifaceted health and longevity effects of CR.

2.9. Metabolic effects of aging and CR

Aging produces a decline in the autophagic and apoptotic turnover of cells, organelles, membranes, carbohydrates, and proteins (Bergamini, 2006; Donati, 2006). It also decreases the expression and capacity of enzymes required for mobilizing protein for the production of metabolic energy (Dhahbi et al., 1999, 2001; Spindler, 2001; Hagopian et al., 2003b,a; Dhahbi and Spindler, 2003; Spindler et al., 2003; Spindler and Dhahbi, 2007). During the postabsorptive state, when blood insulin and glucose levels wane, and glucagon, glucocorticoids and catecholamines increase, most tissues begin to utilize amino acids derived from protein turnover to generate energy via the tricarboxylic acid (Krebs) cycle. This drives the degradation of glycogen and the autophagic degradation of proteins, organelles, and membranes. The glucagon/insulin ratio is a major regulator of autophagy in vivo (Meijer and Codogno, 2006). Insulin represses autophagy. During the postabsorptive period, the high glucagon/insulin ratio activates autophagy (Meijer and Codogno, 2006). Amino acid catabolism leads to the transport of carbon and nitrogen to the liver, mostly as glutamine and alanine, for synthesis into glucose and for nitrogen disposal as urea. These changes are consistent with the decrease in whole body protein turnover found with age in rodents (Goodman et al., 1980; Lewis et al., 1985; el Haj et al., 1986; Goldspink et al., 1987; Merry et al., 1987, 1991, 1992). Decreased macromolecular turnover may underlie the age-related accumulation of oxidatively and otherwise damaged protein. This decrease may exacerbate the effects of the oft-reported age-related increase in oxidant production by isolated muscle mitochondria (e.g. Bejma and Ji, 1999; Mansouri et al., 2006).

Broadly speaking, CR appears to lead to increased catabolism of protein and lipid derived from proteolysis, autophagy, and the autophagic and apoptotic death of mitotically competent cells to generate substrates for energy generation. For example, CR stimulates autophagy in rat liver (Cavallini et al., 2001; Donati et al., 2001; Bergamini et al., 2007). This is apparent in the genetic and enzymatic shift toward increased protein, lipid, and organelle degradation and disposal (turnover) (Feuers et al., 1989; Tillman et al., 1996; Dhahbi et al., 1999, 2001; Cao et al., 2001; Spindler, 2001; Dhahbi and Spindler, 2003; Hagopian et al., 2003b,a; Spindler et al., 2003; Tsuchiya et al., 2004; Spindler and Dhahbi, 2007). CR increases the enzymatic capacity of the muscle for the mobilization of carbon for glucose synthesis, and the disposal of nitrogen as urea (Tillman et al., 1996; Dhahbi et al., 1999, 2001; Cao et al., 2001; Spindler, 2001; Dhahbi and Spindler, 2003; Spindler et al., 2003). Together, these changes enhance energy

generation from protein in the tissues, and gluconeogenesis in the liver to supply glucose for energy production for the brain and other organs, and disposal of the nitrogen produced from protein turnover as urea. We found very similar changes in metabolic enzyme expression in Ames dwarf mice, which are also long-lived (Tsuchiya et al., 2004).

These metabolic responses to CR are apparent in their effect on organ physiology. For example, CR decreases the rate of DNA and protein synthesis, and decreases the RNA content of the liver, kidney, heart, and small intestine in rats (Lewis et al., 1985; Merry and Holehan, 1985; el Haj et al., 1986; Goldspink et al., 1987; Merry et al., 1987, 1991, 1992; Merry and Holehan, 1991; Nikolich-Zugich and Messaoudi, 2005). After the initiation of either CR or fasting, mitotically competent tissues such as liver and lung undergo a profound, rapid, and reversible loss of cells (via necrosis, apoptosis, and/or autophagic cell death), proteins, and lipids (Kouda et al., 2004; Massaro et al., 2004). Intracellular turnover in metazoans involves degradation of cytoplasmic and nuclear proteins by cellular calpains (Sorimachi et al., 1997) and the proteasome (Myung et al., 2001), degradation of mitochondria by mitochondrial proteases (Bakala et al., 2003), autophagic degradation of membranes, mitochondria, ribosomes, ER, and peroxisomes (Bergamini, 2006; Donati, 2006; Kadowaki et al., 2006), and cellular turnover initiated by apoptotic, necrotic, and autophagic cell death (Jin and El Deiry, 2005). Nutritional stress increases the rate of these processes (Finn and Dice, 2006). For example, fasting a rat for 48 h reduces liver weight by half and liver proliferative index by 85%, while increasing its apoptotic index by 2.5-fold (Kouda et al., 2004). The number of lung alveoli in mice is reduced by 35% after 72 h of 33% CR (Massaro et al., 2004). Fifteen days of CR reduces alveolar number by 45% (Massaro et al., 2004). Refeeding for 72 h fully restores alveolar number (Massaro et al., 2004). Thus, CR appears to tip the regulatory balance in some mitotic tissues toward apoptosis and the degradation of cellular carbohydrate, protein, and lipid. Despite this increase in macromolecular degradation and decreased rates of macromolecular synthesis and cell division, LTRC animals are able to maintain their organ mass by balancing these effects to produce higher rates of protein turnover and smaller visceral organ sizes (Weindruch and Sohal, 1997). LTRC (30%) reduces the weight of the heart, liver, kidney, spleen, prostate, and skeletal muscle of rats by 25–50% (Weindruch and Sohal, 1997). This turnover may drive many of the beneficial effects of CR on health and lifespan.

2.10. CR and autophagy

The enhancement of autophagy by CR may be necessary, but not sufficient for its effects on lifespan. Autophagy is induced by nutrient deprivation (Mizushima and Klionsky, 2007). The rate of autophagy declines with age in essentially all tissues (Reznick and Gershon, 1979; Cuervo et al., 2005). CR enhances autophagy (Cavallini et al., 2001; Donati et al., 2001). Further, in wild-type and long-lived worm mutants, mutational reduction of autophagy shortens lifespan (Melendez et al., 2003; Hars et al., 2007). In contrast, enhanced expression of the autophagy-related 8a gene, a rate limiting autophagy gene, in older *Drosophila* brains, extends average adult lifespan by 56% and promotes resistance to oxidative stress (Simonsen et al., 2008). In *C. elegans*, mutational inactivation of essential autophagy-related genes abolishes the extended longevity phenotype of eat-2 mutants, which are a model of CR (Morck and Pilon, 2006; Jia and Levine, 2007; Hansen et al., 2008; Toth et al., 2008). These results suggest that autophagy is required for the CR-related longevity response. However, enhanced autophagy does not appear to be sufficient for lifespan extension. In *C. elegans*, inhibiting genes required for autophagy blocks the longevity effects of CR (Hansen et al., 2008). However, lifespan

extension by Daf-2 (IR/IGFR) longevity mutations requires both autophagy and DAF-16/FOXO. Mutation of daf-16 leaves autophagy active, but blocks lifespan extension by the Daf-2 longevity mutations (Hansen et al., 2007).

The role of autophagy in the effects of CR on longevity in mammals remains unclear at present. However, the importance of turnover and renewal, its decline with age, and its enhancement by CR strongly suggests that autophagy also is central to the longevity effects of CR in mammals. Pharmacological enhancement of autophagy may prove therapeutic in protein-aggregation related neurodegenerative disorders such as Huntington's disease and some forms of Parkinson's disease (see Sarkar et al., 2009, and reference therein).

2.11. CR and glucocorticoids

Glucocorticoids may contribute to the anticancer effects of CR (Masoro, 1995; Nelson et al., 1995). CR transiently increases the diurnal level of free plasma corticosterone in mice and rats (Sabatino et al., 1991; Klebanov et al., 1995; Han et al., 2001). Both 20 and 40% CR significantly decrease the incidence and multiplicity of skin papillomas initiated with 7,12-dimethylbenzanthracene (DMBA) and promoted with 12-O-tetradecanoylphorbol-13-acetate (TPA) in female, SENCAR mice (Stewart et al., 2005). Adrenalectomy partially reverses this inhibition of papilloma multiplicity and incidence. Corticosterone supplementation of the CR-adrenalectomized mice restores much of the ability of CR to reduce papilloma incidence and multiplicity. Glucocorticoid replacement restores the effectiveness of CR at inhibiting DMBA- and TPA-induced skin, and DMBA-induced pulmonary carcinogenesis in adrenalectomized mice (Pashko and Schwartz, 1992; Pashko and Schwartz, 1996). Thus, at least some of the anticancer effects of CR may require glucocorticoids.

The reason for this dependence on glucocorticoids is not known. However, glucocorticoids do suppress cellular proliferation and enhance apoptosis in some cell types, including osteoblasts, lymphocytes, and keratinocytes (Weinstein, 2001; Budunova et al., 2003; O'Brien et al., 2004; Herold et al., 2006). Thus, elevation of glucocorticoids in combination with suppression of sex steroids, insulin and IGF1 may cooperate to produce a part of the anticarcinogenic activity of CR.

2.12. Cardiovascular effects of CR

Aging impairs cardiovascular capacity, contractility, and diastolic and systolic function (McGuire et al., 2001). In rodents and humans, three major age-associated changes markedly affect myocardial performance. First, the development of myocardial fibrosis, a hallmark of cardiac aging in both humans and rats, is initiated by cellular necrosis and apoptosis (Eghbali et al., 1989; Anversa et al., 1990). Cell death induces reparative interstitial and perivascular collagen deposition, which plays a key role in the development of fibrosis in aged human and rodent hearts (De Souza, 2002). Fibrosis decreases cardiac distensibility and increases diastolic pressure, impairing coronary hemodynamics and lowering coronary reserve (Janicki, 1992; Varagic et al., 2001). Second, the decline in the number of cardiomyocytes with age due to necrosis and apoptosis is followed by compensatory myocyte hypertrophy (Colucci, 1997; Lushnikova et al., 2001). This remodeling and the weakening and bulging of the ventricular wall is the most common cardiac manifestation of aging (Lakatta, 2000). Remodeling necessitates increased atrial and ventricular filling pressure. These general age-related changes appear to underlie cardiac arrhythmias, dysfunction, and failure. Third, an age-related impairment in mitochondrial bioenergetics appears to contribute to myocardial stiffness, apoptosis, atrophy and

compensatory hypertrophy (Moreau et al., 2004; van Raalte et al., 2004).

CR is known to be a highly effective means of reducing the incidence and increasing the mean age of onset of cardiovascular diseases (Wagh and Stone, 2004). Approximately 40% of male C57BL/6 mice develop cardiomyopathy by 1000 days of age (Turturro et al., 2002). Aging produces extensive changes in cardiac gene expression in mice, including changes consistent with a metabolic shift from fatty acid to carbohydrate metabolism, increased expression of extracellular matrix genes, and reduced protein synthesis (Lee et al., 2002). LTCR produces changes in gene expression in the heart consistent with preserved fatty acid metabolism, reduced DNA damage, decreased innate immune activity, and cytoskeletal reorganization (Lee et al., 2002). We found that the initiation of CR in older mice rapidly reduces the expression of genes associated with extracellular matrix and cytoskeletal structure and dynamics, cell motility, and inflammation (Dhahbi et al., 2006). The initiation of CR also increases the expression of genes associated with PPAR α signal transduction and fatty acid metabolism for energy production (Dhahbi et al., 2006). Beginning CR in older mice reduces natriuretic peptide precursor type B expression along with the expression of a number of forms of collagen, and reduces perivascular collagen deposition in the heart (Dhahbi et al., 2006). LTCR also preserves smaller cardiomyocytes in the left ventricle of the heart of old-LTCR mice, suggesting reduced age-related cell death and hypertrophy (Dhahbi et al., 2006; Spindler and Dhahbi, 2007). Together these changes are consistent with a rapid CR-related reduction in the forces leading to the development of myocardial fibrosis, tissue remodeling, and hemodynamic stress.

2.13. Immunological effects of CR

The immune system declines with age, which leads to an impaired ability to respond to vaccinations and infections (McElhaney, 2005; Effros, 2007). Elderly people are particularly susceptible to influenza, with 80–90% of mortalities from infection with influenza virus occurring in individuals aged 65 years and older (Trzonkowski et al., 2009). Elderly individuals also suffer more frequently from autoimmunity (Yung and Julius, 2008).

The data regarding the effects of age on innate immune function are contradictory. Some find an age-related decline in the function of neutrophils, macrophages, and natural killer cells, while other studies detect no such changes (Gomez et al., 2008). However, studies in mice and humans have shown that the adaptive immune system is deregulated by aging (reviewed by Dorshkind et al., 2009). Thymic involution reduces the number of naïve T-cells produced with age (Jamieson et al., 1999). Also, B-cell production in the bone marrow is reduced with age (Jamieson et al., 1999; Johnson et al., 2002; Miller and Allman, 2003; Van der et al., 2003). Lymphocytes from older humans have impaired ability to undergo immunoglobulin class-switching recombination (Frasca et al., 2008). There also is an age-related decline in CD4⁺ T helper cell function which may contribute to this impairment (Haynes and Swain, 2006). CD4⁺ T cells, but more so CD8⁺ T cells, undergo clonal expansion in the elderly, limiting the repertoire of antigens to which the resident T cells can respond and blocking the niches that otherwise would be colonized by newly formed immune cells (Haynes and Swain, 2006).

CR can inhibit the age-related decline in immune function in mice, nonhuman primates, and humans. While aging causes a shift from naïve to memory cells, CR increases the number of naïve T cells and the diversity of the T-cell repertoire (Miller, 1996; Pawelec et al., 1999; Nikolich-Zugich and Messaoudi, 2005; Messaoudi et al., 2006). CR also retards the age-related decline in antigen presentation, T-cell proliferation, and antibody produc-

tion in response to influenza vaccination (Effros et al., 1991; Pahlavani, 2000). However, CR also can increase mortality in mice in response to influenza virus infection, probably due to lack of energy reserves (Ritz and Gardner, 2006). Further, in contrast to the benefits of CR initiated during early adulthood, CR initiated in young male rhesus monkeys accelerates the loss of naïve T cells, decreases T-cell repertoire diversity, and increases the frequency of T-cell clonal expansions (Messaoudi et al., 2008), predisposing them to a reduced immune response after infection (Messaoudi et al., 2004). CR initiated in old rhesus monkeys produces lymphopenia and decreases T-cell proliferative capacity, suggesting that CR initiated at older ages can have adverse effects in primates (Messaoudi et al., 2008). Thus, little is known about how CR will affect the immune system of humans, especially elderly humans.

2.14. Neurological effects of CR

Neuronal loss promotes the cognitive, sensory, and motor impairments found with age (Kuhn et al., 1996). CR appears to reduce neuronal damage in response to toxins and other stressors, protect against neurodegenerative diseases, and increase neurogenesis and synaptic plasticity, even in old animals (Gillette-Guyonnet and Vellas, 2008). Aging is accompanied by a reduction in synaptic contacts, synaptic strength, neural plasticity, brain neurogenesis (Burke and Barnes, 2006; Kuhn et al., 1996), and spinal cord densities and neurogenesis (Burke and Barnes, 2006; Segovia et al., 2006; Fontan-Lozano et al., 2008). Aging alters the expression of the neurotransmission related genes N-methyl-D-aspartate receptor, brain derived neurotrophic factor, Trk-B, and α -synuclein (Mattson et al., 2001). For some of the genes, CR counteracts these changes (Mattson et al., 2001).

The cells of the aging brain experience increased levels of oxidative stress (Serrano and Klann, 2004; Zecca et al., 2004; Martin et al., 2006), damaged protein (Gray et al., 2003; Trojanowski and Mattson, 2003; Martin et al., 2006), and damaged DNA (Lu et al., 2004; Kyng and Bohr, 2005). Aging also leads to changes in the normal brain that are similar to, but less severe than, the changes found in some age-related neurological diseases. These changes include amyloid- β accumulation (Trojanowski and Mattson, 2003), as found in Alzheimer's disease; α -synuclein accumulation and dopamine depletion in substantia nigra neurons, as found in Parkinson's disease (Grondin et al., 2002); Cu/Zn-SOD accumulation in motor neurons, as found in amyotrophic lateral sclerosis (Rakhit et al., 2004); and reduced BDNF levels, as found in Alzheimer's disease and Huntington's disease (Zuccato et al., 2003; Mattson et al., 2004).

LTCR either reduces the rate of damage, or reverses some of the age-related degenerative changes described above (Idrobo et al., 1987; Ingram et al., 1987; Stewart et al., 1989; Pitsikas et al., 1991; Pitsikas and Algeri, 1992; Eckles et al., 1997; Eckles-Smith et al., 2000). LTCR increases neurotrophic factor levels and attenuates neurochemical and behavioral deficits in a primate model of Parkinson's disease (Maswood et al., 2004). CR protects neurons and improves the functional outcome in rodent and monkey models of stroke, Alzheimer's, Parkinson's, and Huntington's diseases (Mattson et al., 2004, and references therein). CR attenuates Alzheimer's disease type brain amyloidosis in Squirrel monkeys (*Saimiri sciureus*) (Qin et al., 2006). CR also increases the levels of brain-derived neurotrophic factor and heat-shock proteins in neurons and stimulates neurogenesis in the hippocampus (Mattson et al., 2004); and references therein). Neurons of LTCR rats and mice are more resistant to oxidative, metabolic, and excitotoxic stressors than those of controls (Reviewed in Martin et al., 2006). CR induces analgesia in acute and chronic models of pain (los Santos-Arteaga et al., 2003; Hargraves and Hentall, 2005).

Late-onset, STCR can bring about many of the beneficial neurological effects found in animals subjected to LTCR. CR initiated late in life can reverse the age-related downregulation of neural cell adhesion molecule and polysialylated-neural cell adhesion molecule in various brain regions, and reverse the age-related upregulation of astrocytic glial fibrillary acidic protein in old rats (Kaur et al., 2008). These results suggest that late-onset STCR can have beneficial effects on neuroplasticity. These results may translate to humans. High caloric intake induced obesity increases the risk of age-related cognitive decline (Knecht et al., 2008). Just 3 months of 20% CR in elderly humans significantly improves verbal memory scores, in concert with a decrease in fasting plasma levels of insulin and high sensitive C-reactive protein (Witte et al., 2009).

2.15. Genic effects of CR

Here we focus on results of large scale gene-expression studies in mammals. Genetic studies in lower eukaryotes have been reviewed elsewhere (Mair and Dillin, 2008). Large-scale microarray studies have allowed a number of important broad conclusions to be drawn regarding aging and the effects of CR in mammals. First, aging appears to shift tissues and cells toward patterns of gene expression consistent with enhanced inflammation, stress, and the age-related pathologies of each tissue type studied (e.g. Lee et al., 1999, 2000, 2002; Cao et al., 2001; Kayo et al., 2001; Dhahbi et al., 2004, 2006; Tsuchiya et al., 2004; Spindler and Dhahbi, 2007). Second, the majority of the genic responses of tissues to CR appear to be tissue- and cell-specific, and tailored to resisting or reversing the characteristic age-related dysfunctions of specific tissue or cell-types (e.g. Lee et al., 1999, 2000, 2002; Cao et al., 2001; Kayo et al., 2001; Dhahbi et al., 2004, 2006; Tsuchiya et al., 2004; Spindler and Dhahbi, 2007).

Finally, there appears to be broad differences in the way mitotically competent and “postmitotic” tissues respond to CR. The genic responses found are consistent with other evidence cited elsewhere in this article suggesting that mitotically competent tissues shift toward increased susceptibility to apoptotic cell death, while postmitotic tissues shift toward increased cellular repair, and stress resistance (Spindler and Dhahbi, 2007). For example, in heart we and others have found that CR produces gene expression and histochemical changes associated with reduced fibrosis, tissue remodeling, apoptosis and blood pressure, and alterations in signal transduction associated with enhanced lipid catabolism for energy production and contractility (Edwards et al., 2003; Gonzalez et al., 2004a; Dhahbi et al., 2006). In contrast, in liver, CR produces changes in gene expression and enzymology associated with the differentiated functions of the liver, including induced fatty acid oxidation, gluconeogenesis, detoxification, and apoptosis of damaged cells (Reviewed in Spindler and Dhahbi, 2007). Likewise, CR reduces gene expression associated with glycolysis and fatty acid biosynthesis. Importantly, the Ames longevity mutation produces many of these same effects (Tsuchiya et al., 2004; Spindler and Dhahbi, 2007).

2.16. Hypothermia and CR

CR lowers the mean body temperature of homeotherms such as mice, rats, monkeys, and humans (Weindruch and Walford, 1988; Walford and Spindler, 1997; Walford et al., 2002). This decrease in temperature is likely to be due, in part, to a lowering of triiodothyronine (T_3) levels. Long-lived mutant mouse strains have reduced serum T_3 levels and core body temperatures, including the Ames, Snell, and the growth hormone receptor/binding protein knockout dwarf mice (Hauck et al., 2001; Bartke and Brown-Borg, 2004). However, thermal regulation is more

complex than just thyroid hormone levels (Swoap, 2008). In mice, the decrease in temperature in response to 40% CR can vary from 1.5 to 5.8 °C below normal, depending on mouse strain, indicative of its multigenic character (Rikke et al., 2003).

Lowering the body temperature of poikilothermic vertebrates, such as annual fish, by 3–5 °C can substantially prolong their lifespans (Liu and Walford, 1966; Liu and Walford, 1970; Liu and Walford, 1972). Further, the lifespans of ectothermic animals such as *C. elegans* (Klass, 1977) and *Drosophila* (Lamb, 1968) are inversely related to environmental temperature. This seems to strongly suggest that the decrease in body temperature and the longevity effects of CR are causally related, at least in poikilotherms. However, in these animals, CR and temperature actually may use different pathways to regulate longevity (Miquel et al., 1976; Mair et al., 2003).

Body temperature appears to contribute to the health and longevity benefits of CR in mice (Turturro and Hart, 1991; Walford and Spindler, 1997). Support for this idea has come more recently from studies of transgenic mice overexpressing uncoupling protein 2 only in hypocretin neurons (Hcrt-UCP2) (Conti et al., 2006). These mice have a 0.3–0.5 °C reduction in core body temperature, and a 12% increase in male and a 20% increase in female median lifespan, independent of caloric consumption (Conti et al., 2006). Another line of evidence supporting a link between body temperature and lifespan comes from a study of C57BL/6 mice housed either at the normal housing temperature of 20–23 °C, or at 30 °C (Koizumi et al., 1996). When housed at the normal temperature, 40% CR produces a 1 °C decrease in core body temperature, while housing at 30 °C prevents this decrease (Koizumi et al., 1993; Jin and Koizumi, 1994). Housing the mice at 30 °C reversed the life-extending effects of CR, largely by decreasing its anti-lymphoma action (Koizumi et al., 1996). The reduction of the anti-lymphoma action may involve loss of the general inhibitory action of CR on cellular proliferation. Housing LTCR Fischer 344 rats at 30 °C largely cancels the normal CR-related reduction in cellular proliferation found in the jejunum, epidermis, and lung of these animals (Jin and Koizumi, 1994). Housing C57BL/6 female mice at 30 °C also antagonizes the suppressive effect of LTCR on white blood cell counts (Koizumi et al., 1993). These counts are considered indicative of the cellular proliferation rates in the bone marrow. Thus, decreasing core body temperature may be sufficient to extend lifespan and be required for the antiproliferative and anticancer effects of CR.

2.17. Exercise and CR

The relationship between exercise and biomarkers of health in humans is discussed above. The question of whether exercise will produce effects similar to those of CR has been addressed in rodents by Holloszy and his colleagues (Holloszy and Smith, 1987; Holloszy and Schechtman, 1991; Holloszy, 1992; Holloszy, 1997). Exercise reproduces a number of the same physiological effects as LTCR in rats. Exercised rats are smaller and have less fat than sedentary animals fed the same number of calories. Young rats, about 4 months of age, will run voluntarily almost 8 kilometers per day if a running wheel is placed in their cages. As they age, their running distances decline. But, if their food intake is reduced by 8% of ad libitum consumption, their running behavior declines much more slowly, and will continue at reduced levels even into extreme old age (Holloszy et al., 1985). The runners are much leaner and smaller overall than sedentary control rats fed the same number of calories as the runners. However, while running significantly increased the mean lifespan of the rats by about 10%, it did not increase their maximum lifespan (Holloszy and Schechtman, 1991; Holloszy, 1997). CR is capable of increasing both the mean and maximum lifespan of rats.

The meaning of these results remains unclear. Gerontologists often aver that the extension of average and maximum lifespan are mechanistically different. They often conjecture that extension of maximum lifespan means that CR slows something termed *the underlying rate of aging*. However, CR can extend the maximum and average lifespan of mice by slowing the rate of tumor growth (Dhahbi et al., 2004; Spindler, 2005). It seems unlikely that very many gerontologists would regard a regimen of cancer chemotherapy as “slowing the underlying rate of aging”. Thus, the possible mechanistic differences between the extension of average and maximum lifespan remain to be defined.

2.18. Adiposity and the health effects of CR

There is a direct relationship between adiposity, especially visceral fat mass, and mortality in humans (Takata et al., 2007; Fair and Montgomery, 2009). CR clearly reduces body fat, especially visceral fat, in mice, rats, rhesus and cynomolgus monkeys, and humans (Reviewed in Masoro, 2005). Therefore, adiposity may be important in the longevity effects of CR. Initially, several studies seemed to suggest that reduced fat mass is not required for the longevity effects of CR (Bertrand et al., 1980; Harrison et al., 1984). The strongest piece of evidence came from a study in which ob/ob mice, which are leptin-deficient and therefore hyperphagic and obese, were compared to congenic lean mice lacking the ob/ob mutation. CR ob/ob mice live twice as long as ad libitum-fed lean mice, even though the ob/ob mice have over twice the adipose weight of the congenic lean mice (Harrison et al., 1984). These results argue that adipose fat mass is not a key determinant of longevity in CR mice. This conclusion is consistent with a previous longitudinal study that found that body weight throughout life is not correlated with the lifespan of ad libitum fed rats (Bertrand et al., 1980).

More recent studies using fat-specific insulin-receptor knock-out (FIRKO) mice, in which the IR has been specifically knocked out in adipose tissue, suggest that adipose may have an important effect on lifespan, perhaps through the action of a secreted factor. FIRKO mice have increased median (18%) and maximum (14%) lifespan compared with phenotypically normal littermate controls (Bluher et al., 2003). The knockout renders adipose cells insulin resistant, but the mice are insulin sensitive and lean despite food intakes equivalent to those of controls. The mice also have increased systemic insulin sensitivity and resistance to diabetes, even when fed a high-fat diet. The authors suggest that these effects may be due to the reduction in fat mass. Thus, a systemically-active secretory product from adipose tissue may be responsible. As discussed by Russell and Kahn (2007), leptin, which is secreted by adipose, is unlikely to be this factor, since leptin levels in FIRKO mice are similar to those in controls. Adiponectin, which is secreted by adipose, is a possibility, since its level increases in CR rats and humans (Zhu et al., 2004; Weiss et al., 2006), and it increases in FIRKO mice (Bluher et al., 2002). Other possible factors include steroid hormones, Russell and Kahn (2007). Adipose both synthesizes and metabolizes sex steroids (Belanger et al., 2002). The diffusible factor also could be an unidentified adipokine or small molecule.

2.19. Human BMI, morbidity, and mortality

CR men and women undergo many of the same metabolic adaptations that occur in CR rodents and monkeys, including decreased metabolic, hormonal, and inflammatory risk factors for diabetes, cardiovascular disease, and cancer (see above for references). Indeed, most published studies focus on the relationship between BMI and disease-related mortality, because caloric intake is difficult to measure in population based studies. Most of

these studies find a positive association between elevated BMI (obesity) and risk of mortality from all causes, including cancer and cardiovascular disease, and reduced BMI with a lower risk of mortality from cardiovascular disease and many types of cancer (reviewed in Takata et al., 2007; Fair and Montgomery, 2009). For example, a prospective cohort study of Swedish women diagnosed and treated for anorexia prior to age 40 found that they had about half the risk of breast cancer relative to age-matched controls (Michels and Ekblom, 2004). A prospective cohort study of Canadian women who consumed in excess of 2,406 kcal/day had a significantly higher risk of breast cancer than women who consumed less than 1,630 kcal/day (Silvera et al., 2006). Data from the Shanghai Breast Cancer Study showed that women with higher BMIs or higher calorie intakes and lower levels of physical activity were at increased risk of breast cancer (Malin et al., 2005). In both case-control and cohort studies, obesity has been consistently associated with higher risk of colorectal cancer for both men and women (Slattery et al., 1997; Fair and Montgomery, 2009). Colon cancer risk in men with a BMI > 30 is increased by up to 80% (McMillan et al., 2006). A meta-analysis of 14 studies was used to estimate that excess weight accounts for 39% of cancers of the endometrium and approximately 25% of cancers of the kidney and gallbladder in both sexes (Bergstrom et al., 2001). An analysis of data from the first National Health and Nutrition Examination Survey (NHANES), and the NHANES Epidemiologic Follow-up Study encompassing 14,407 participants in the age group of 25–74 years found that all neoplasms in women decreased with decreasing BMI from approximately a BMI of 29 to a BMI of 17 (Sunder, 2005). A similar decrease was not found for men. Together, the results suggest that lower body weight, and lower caloric intake is associated with reduced mortality from all causes, including cancer and cardiovascular disease.

However, a number of such studies also show that there is an association between low BMI and increased mortality from all causes, including cardiovascular disease, and cancer in the middle aged and elderly. For example, a community-based longitudinal study of the association between BMI and all-cause mortality in 80–84 year olds found that total mortality in the overweight group was 52% less than that in the normal weight group (Takata et al., 2007). In this study, underweight individuals had all-cause mortality 3.9 times higher, and mortality from cancer 17.7 times higher than overweight individuals. Further, mortality from cardiovascular disease was 4.6 times higher in the underweight compared to the normal-weight subjects. These results are in agreement with studies of individuals aged 60 and older (Janssen et al., 2005), adults and the elderly (Pitner, 2005; Shahar et al., 2005), those 65–84 years of age (Sergi et al., 2005), those averaging 71 years of age (Wassertheil-Smoller et al., 2000), those averaging 73 years of age (Corrada et al., 2006), and those of middle-age (Flegal et al., 2005; Hayashi et al., 2005; Gu et al., 2006). Low BMI is also found associated with greater mortality and/or morbidity from cardiovascular disease and cancer (Wassertheil-Smoller et al., 2000; Cui et al., 2005; Hu et al., 2005; Chen et al., 2006).

A number of investigators have found “U shaped” survival versus BMI curves, suggesting that being either underweight or overweight increases the risk of mortality. For example, the estimated hazards ratio for individuals of 50–75 years of age is at a minimum from a BMI of 25.7 to 28.9 for men, and from 24.5 to 29.0 for women (Sunder, 2005). This range is higher than typically considered healthy. A population study of Chinese adults found that a BMI from 23.0 to 23.9 had a relative risk of all cause mortality of 1.00, and BMIs which were higher or lower had an increased risk of mortality from all causes (Gu et al., 2006). One possible interpretation of these results is that low BMI among adults and the elderly leads to reduced resistance to the stress of disease, or predisposes them to mortality from disease.

The studies discussed above challenge the idea that human CR will lead to extended longevity. However, there are a number of possible confounds to interpretation of these studies. Normal weight or overweight people who survive into old age may be less susceptible to obesity related diseases. The quality of the diets consumed by the low BMI individuals are difficult to assess, and may lack nutrients important to longevity. The lower weight individuals in the studies are not CR because their caloric intake reflects their individual ad libitum set-points, and not a reduction from that set-point. Still, the absence of a verifiable 160 year old lean human is a challenge to the view that human CR is likely to lead to robust lifespan extension.

2.20. CR insulin/IGFI signaling and human longevity

The potential role of IGFI signaling in human longevity is far from clear. It is possible that the effects of CR may not be readily detectable because of the dependence of serum IGFI levels on diet composition in humans. There is overwhelming evidence that IGFI and IGF1 signaling are important to lifespan in metazoans, as reviewed above. Therefore, the effects of CR on serum IGFI levels may be of key importance to its potential longevity effects in humans. However, neither the Biosphere nor the CALERIE studies reported a decrease in serum IGFI or the IGFI to IGF1 ratio in humans after CR diets lasting from six months to two years (Walford et al., 2002; Holloszy and Fontana, 2007; Redman and Ravussin, 2009). In contrast, Fontana et al. (2006a, 2008) found that total and free IGFI concentrations are significantly lower in moderately protein-restricted CR individuals. In a recent study, reducing protein intake from an average of 1.67 g/kg body weight to 0.95 g/kg body weight per day for 3 weeks in six volunteers already practicing CR reduced serum IGFI from 194 to 152 ng/ml (Fontana et al., 2008). Thus, unlike rodents, humans may need to reduce both protein and calorie intake to lower their serum IGFI levels and their IGFI to IGF1 ratios.

However, in contrast to the data from worms, flies and mice suggesting that lower IGFI levels promote longevity, reduced IGFI levels may not be beneficial to health and longevity of humans. Human IGFI and/or GH deficiency is associated with growth disorders, glucose dysregulation, increased risk of cardiovascular disease and atherosclerosis, and reduced life expectancy (Arai et al., 2009; Besson et al., 2003). Studies of a cohort of 252 centenarians found that the lowest tertile of IGFI and IGF1 serum concentrations were associated with increased mortality (Arai et al., 2008).

Higher serum IGFI levels appear to be associated with greater health and longevity. Centenarians appear to have higher plasma IGFI/IGF1 ratios than those found in control subjects of 75–99 years of age, even though IGFI levels declined with age in both groups (Paolisso et al., 1997). Higher serum levels of IGFI also are associated with the ability of women of all ages to recover and function better after hip fracture (Di Monaco et al., 2009). Further, the anabolic activity of IGFI in tissues, including muscle and bone, appears to be beneficial to the elderly (Lytras and Tolis, 2007). Recombinant human IGFI has been used in the treatment of type 2 diabetes to improve insulin sensitivity and glycemic control (Abbas et al., 2008). IGFI may be a protective factor for the vasculature, and be beneficial for treatment of chronic heart failure (Abbas et al., 2008). Interventions that elevate IGFI levels in the brain appear to improve cognitive functioning in deficient individuals, such as the elderly (Aleman and Torres-Aleman, 2009).

On the negative side, higher levels of IGF-I are associated with increased risk of some types of cancer. For example, a meta-analysis of published studies confirmed that elevated circulating levels of IGFI are positively associated with prostate cancer risk, a common cancer among elderly men (Rowlands et al., 2009). GH-

induced IGFI is required for estrogen and progesterone-mediated induction of mammary gland development and maintenance (reviewed in Kleinberg et al., 2009). However, there is growing consensus that the elevation of one or both of these hormones also can drive progression from normal mammary development to the development of precancerous mammary lesions (Kleinberg et al., 2009).

In contrast to the studies reviewed above, some data support the idea that lower IGFI activity may be beneficial for human longevity. A study of a cohort of Ashkenazi Jewish centenarians and their offspring found that reduced stature and elevated IGFI levels were present in 35% of the female (but not the male) offspring of centenarians (Suh et al., 2008). IGFI receptor mutations were found in transformed lymphocytes from 2.3% of the centenarians (Suh et al., 2008). It was proposed that these mutations reduce receptor signaling, leading to a compensatory increase in serum IGFI levels (Suh et al., 2008). In another study, individuals who carry certain genotype combinations at the IGFI receptor and PI3KCB genes appear to have longer lifespans and higher free plasma levels of IGFI, perhaps due to reduced IGFI receptor activity (Bonafe et al., 2003). These results are indirect, but consistent with the low levels of cancer found among centenarians (Salvioli et al., 2009). Elevated IGFI receptor signaling is associated with higher rates of carcinogenesis (see Section 2.8.2). Thus, it is not clear whether reducing serum IGFI levels via combined protein and caloric restriction will produce a net positive or a net negative effect on human life span.

2.21. The search for longevity therapeutics

As discussed above, there are many published epidemiological and clinical studies suggesting that CR decreases the incidence of cardiovascular disease, type 2 diabetes, and cancer in humans. However, also as reviewed above, there is much we do not know about how low calorie diets and low BMI affect human longevity, especially in the elderly. Despite these considerations, the sales of weight reduction products produce billions of dollars in profits for companies every year, although none of these products are effective in the long run. Even in mice, hunger induced by a CR diet does not diminish over time (Hambly et al., 2007). Family studies demonstrate that tendencies toward obesity or thinness are largely inherited (Maes et al., 1997). Twin and adoption studies also indicate that weight is largely genetically determined (Stunkard et al., 1986, 1990; Sorensen et al., 1989). Thus, it is unlikely that we will avail ourselves of the benefits of being even the “ideal weight” specified by the Metropolitan Height and Weight Tables for Men and Women (Anonymous, 1979). Because humans are not experimental animals maintained in clean, low stress environments, it is not clear if the potential benefits of human CR will be greater than the downsides. However, mild CR in obese humans is very likely to lead to improved health and longevity.

Despite the limitations, the existence of multiple dietary interventions and a number of natural mutations, gene knockout and transgene overexpressing mice with extended longevities suggests that druggable therapeutic targets exist in mammals (Brown-Borg et al., 1996; Zhou et al., 1997; Coschigano et al., 2000; Flurkey et al., 2001; Holzenberger et al., 2003; Kurosu et al., 2005; Taguchi et al., 2007; Selman et al., 2008). However, the identification of therapeutics for these targets continues to be a challenge. There have been numerous attempts to use surrogate assays to identify compounds that can reproduce the longevity and health effects of CR and the mammalian longevity mutations. One strategy has been rodent-lifespan assays. Unfortunately the literature produced by this effort is largely confounded and unreliable (Spindler, submitted for publication).

2.21.1. The literature describing compound screening for lifespan effects

We searched for studies reporting lifespan extension in normal, healthy rodents using repeated key word searches of the PubMed database. We identified 65 lifespan studies performed using healthy, long-lived rodents, not including 21 contradictory melatonin lifespan studies (reviewed in Anisimov et al., 2006; Spindler, submitted for publication). Of these studies, 18 report no effect, or a shortening of lifespan (Spindler, submitted for publication); 5 would be difficult to repeat because the preparation and composition of the treatment agents are published in difficult to obtain journals (Kohn, 1971; Emanuel and Obukhova, 1978; Anisimov et al., 1989, 1997; Ghanta et al., 1990); one reports a shortening of lifespan; 3 are uninterpretable due to lack of information regarding dose, route of administration, number or kind of animals used, high early mortality, or unusually short control lifespans; and 29 report results that are possible or likely artifacts of “voluntary” CR (Spindler, submitted for publication). In most of these studies, neither food intake nor body weight were measured or reported. In some cases the authors write that there was no change in body weight or food intake. However, no data are shown, no indication is given of when or how many times during the study the weight measurements were taken, the means and standard deviations of the measurements, or the statistics used. Three of these studies purport to show lifespan extension, but the data are equivocal (Kohn and Leash, 1967; Ferder et al., 1993; Popovich et al., 2003). Other studies suffer from unexplained, high early mortality, mortality due to air-conditioning malfunctions, short control lifespans, or other problems.

Only 2 studies measured food consumption and found lifespan extension (Yen and Knoll, 1992; Caldeira da Silva et al., 2008). Deprenyl (a monoamine oxidase-B inhibitor) and Dinh lang root extract (a traditional medicine used in the Far East) were reported to extend the lifespan of mice (Yen and Knoll, 1992), and dinitrophenol (an uncoupler of oxidative phosphorylation) slightly extended the median and mean lifespan of a normal, but relatively short lived mouse (Caldeira da Silva et al., 2008). Six other treatments extended lifespan and provided data showing that body weight did not change. These 6 treatments are: 2-mercaptoethanol (a hydroxyl radical scavenger and disulfide bond reductant) administered orally to male BC3F1 mice (Heidrick et al., 1984); cornstarch vs. sucrose-containing diets fed to Fischer 344 rats (Murtagh-Mark et al., 1995); deprenyl fed to Syrian hamsters (Stoll et al., 1994, 1997); ginkgo biloba extract (a traditional Asian herbal medicine) administered orally to male Fischer rats (Winter, 1998); green tea polyphenols (some of which are potent inhibitors of fatty acid synthase and thereby cancer cell growth) administered in drinking water to male C57BL/6 mice (Kitani et al., 2007); N-tert- α -phenyl-butyl nitron (a.k.a. PBN, a spintrap antioxidant) fed to C57BL/6J male mice (Saito et al., 1998), and piperoxane (an α 2-adrenoceptor antagonist) administered by injection to Fischer 344 rats (Compton et al., 1995). However, several of these studies may lack sufficient statistical power to reliably detect a small change in body weight due to small sample size. Even a small decrease in caloric consumption can lead to lifespan extension in rodents (Compton et al., 1995; Merry, 2002).

It is common for natural products and pharmaceuticals to induce “voluntary” CR when administered orally to mice. Oral treatment of mice with a 15% kombucha extract (a tea and sugar solution fermented by a symbiotic colony of staphylococci and pseudomonas, and yeast) extends the lifespan of C57BL/6 mice by 5% (males) and 2% (females) (Hartmann et al., 2000). But, kombucha extract is anorectic and reduces the weight of male mice from 30 to 22 g and female mice from 25 g to 21 g, introducing a CR-related confound into interpretation of these data (Hartmann et al., 2000). Two multi-component nutraceutical

mixtures were reported to extend the lifespan of mice (Bezlepkin et al., 1996; Lemon et al., 2005). One combination (containing β -carotene, α -tocopherol, ascorbic acid, selenite, and zinc), fed to male C57BL/6 mice, reportedly extended their lifespan by 9.5% and 16% (mean and maximum, respectively) (Bezlepkin et al., 1996). The other combination (containing 31 components including vitamins and minerals), fed ad libitum to C57BL/6JxSJL hybrid mice, reportedly extended mean longevity by \sim 11% (Lemon et al., 2005). In both studies the authors state that the body weights were similar between treated and control. However, neither specified at what ages the mice were weighed, how many times they were weighed, how long they had been consuming the diets when weighed, their weights, or the statistical test used. We found that the mixture studied by Lemon et al. (2005) is anorectic (P.L. Mote and S.R. Spindler, unpublished). Male C3B6F1 mice fed this supplement mix cold packed in pellets of the chemically defined AIN-93 M diet, fed at 93 kcal per week, restrict their food intake by \sim 5% or more relative to mice not receiving the supplement (S.R. Spindler and P.L. Mote, unpublished observations). Thus, a CR effect cannot be excluded from these published studies, and even appears to be the likely explanation for the lifespan extension observed by Lemon et al.

Notable among the recent lifespan studies published without either controlling food consumption or reporting body weight was the study discussed above in which microencapsulated rapamycin, fed to mice beginning at 600 days of age, appeared to significantly extend the mean and maximum lifespan of both males and females (Harrison et al., 2009). Average lifespan was extended by an average of about 10% in males and about 13% in females. This is a potentially important result because it is the first well supported report of lifespan extension in old mice using a pharmacologic agent.

The use of enfeebled, transgenic, knockout, or short-lived rodent strains; and the use of pro-oxidants, toxins, carcinogens, radiation treatment, and transplanted or induced tumors introduces other confounds into lifespan studies (Spindler, submitted for publication). There is no evidence that compounds that mitigate the effects of such enfeeblments are capable of extending the lifespan of healthy rodents or humans. Studies of enfeebled rodents have not proven helpful for identifying nutrients or drugs capable of extending the lifespans of healthy rodents or humans.

2.21.2. Preferred design for screening longevity therapeutics using rodent lifespan

Review of the studies outlined above led us to design and execute a lifespan study involving 62 treatment groups of mice (Spindler, submitted for publication). These studies observed a number of design parameters which are essential for obtaining meaningful rodent lifespan results (Spindler, submitted for publication). These are: (1) Use of a long-lived, healthy rodent strain, preferably an F1 or further outcrossed strain. (2) Feeding of the diets in measured amounts. The presence of any uneaten food can be noted, and the subsequent day's allotment of food adjusted accordingly. The amount of food fed to the entire study can be reduced if food is left by a treatment group. (3) Feeding of only chemically defined diets, such as the AIN diets, in any study. (4) Use of daily feeding. This ensures that the animals are dosed daily with an effective concentration of the test compounds. (5) Use of a positive control, such as a CR group. Most lifespan studies are published without a positive control. However, negative results are difficult to interpret in the absence of a positive control.

2.21.3. Screening longevity therapeutics using surrogate biomarkers

Another approach we and others have explored for identifying longevity therapeutics is screening of compounds for their ability to reproduce surrogate markers of extended health and longevity.

We used the genome wide, gene expression signature of LTCR as the surrogate marker (Spindler and Mote, 2007). Gene expression patterns appear to be reliable indicators of biological status. For example, the gene expression patterns of primary tumor cells appear to be useful for predicting clinical outcomes such as chemosensitivity, metastases, and survival (Rosenwald et al., 2002; Vasselli et al., 2003). In mice, both the anticancer and the gene-expression response of the liver to CR is rapid (Dhahbi et al., 2004). A similarly rapid, but less extensive response also was found in heart (Dhahbi et al., 2006). We found that 8 weeks of metformin treatment of old mice was superior to even 8 weeks of CR at reproducing the global gene expression signature of LTCR in liver (Dhahbi et al., 2005). As discussed in Section 2.5.3, metformin increases insulin sensitivity, decreases hepatic gluconeogenesis, inhibits the development of metabolic syndrome, may reduce cancer risk in humans and animals, and extends the lifespan of short-lived or enfeebled mice. These results suggest that gene-expression profiling is useful for the identification of potential longevity therapeutics.

Thus, the results of our work reviewed above, and work of others (Corton et al., 2004; Ingram et al., 2006; Miller et al., 2007; Anisimov et al., 2008; Caldeira da Silva et al., 2008; Pearson et al., 2008; Strong et al., 2008) suggest that longevity therapeutics exist, that procedures can be developed for identifying them, and that eventually such therapeutics will be used to extend the healthy human lifespan. When this goal is attained, we will have fulfilled one of the most ancient of human goals (Walford, 1998).

3. Conclusions

We know that it is possible to extend the mean and/or maximum lifespan of mammals by reducing dietary calories, protein, methionine, or tryptophan; or by reducing insulin and/or IGF1 signaling. These interventions also delay the onset of deleterious age-related physiological changes and diseases. At least in mice and *Drosophila*, lifespan extension can be reversibly induced by CR, even in “middle age”. The longevity and health effects of CR are phylogenetically widespread. CR appears to be an adaption to boom and bust cycles in the food supply. Whether human lifespan can be similarly extended by CR or any means remains open to debate. However, studies in humans indicate that CR produces many of the same physiologic, hematologic, hormonal, and biochemical changes produced in species which experience lifespan extension. In humans, nonhuman primates, and rodents, CR provides protection from type 2 diabetes, cardiovascular and cerebral vascular disease, age-related immunological decline, malignancy, hepatotoxicity, liver fibrosis and failure, sarcopenia, systemic inflammation, and DNA damage. It produces intense organelle, lipid and protein turnover and renewal, enhances muscle mitochondrial biogenesis, affords neuroprotection; and extends mean and in some cases maximum lifespan. CR also may increase apoptosis rates in tissues capable of cell replacement and in tumors. The lifespan effects of CR are dose and duration responsive in rodents. Meal frequency appears to have little, if any effect on the lifespan response. In mice, CR rapidly induces powerful antineoplastic effects in the liver and lung, and salutary effects on the physiology of the cardiovascular system. CR induces transient increases in serum glucocorticoid levels, and glucocorticoids appear to be required for at least some of the anticancer effects of CR in rodents. In dogs, CR mitigates the onset of arthritis. CR, amino acid restriction, and disrupted insulin and IGF-I signaling also downregulate thyroid hormones, and reproductive hormones and activity. CR lowers the mean body temperature of homeotherms such as mice, rats, monkeys and humans, and this appears to contribute to its health and longevity effects in mice. Hypothermia also appears to contribute to the

enhanced longevity of long-lived mutant mice. In addition to its effects on the insulin and IGF-I signaling systems, CR may also induce lifespan extension by altering the activity of transcription factors including Sirt1, PGC-1 α , AMPK, and TOR. Paradoxically, low body weight in middle aged and elderly humans is associated with increased mortality. Thus, a caloric intake below that required to maintain a BMI associated with a low relative risk of all cause mortality may be unlikely to extend human maximum lifespan. Enhancement of human maximum lifespan may require pharmaceutical interventions. A number of surrogate assays are currently being applied in the search for authentic longevity pharmaceuticals. The literature claiming to have identified drugs or nutrients which extend the lifespan of rodents are often confounded by design flaws, most commonly, by potential “voluntary” CR. We suggest a number of design parameters which will avoid such confounds in future studies.

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