PERSPECTIVE

How does calorie restriction work?

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For almost 70 years, calorie restriction has been known to extend life span. Despite the extensive physiological characterization of this dietary regimen, the molecular basis for the slowing in aging remains unsolved. Recent findings have pinpointed a few molecular pathways that appear to regulate the aging process. In this review, we propose a molecular model for how calorie restriction works that incorporates these recent findings.

Calorie restriction (CR) refers to a dietary regimen low in calories without undernutrition. It was first noted in the 1930s that food restriction significantly extends the life span of rodents (McCay et al. 1935). This longevity results from the limitation of total calories derived from carbohydrates, fats, or proteins to a level 25%–60% below that of control animals fed ad libitum (Richardson 1985; Weindruch et al. 1986). The extension in life span can approach 50% in rodents (Sohal and Weindruch 1996). CR extends life span in a remarkable range of organisms, including yeast, rotifers, spiders, worms, fish, mice, and rats (Weindruch and Walford 1988). Emerging data show that its effect may also apply to nonhuman primates (Lane et al. 2001).

CR delays a wide spectrum of diseases in different experimental animals; for example, kidney disease, a variety of neoplasias, autoimmune disease, and diabetes (Fernandes et al. 1976; Sarkar et al. 1982; Fernandes and Good 1984; Kubo et al. 1984; Engelman et al. 1990; Shields et al. 1991; Johnson et al. 1997). CR reduces age-associated neuronal loss in most mouse models of neurodegenerative disorders such as Parkinson's disease (Duan and Mattson 1999) or Alzheimer's disease (Zhu et al. 1999). However, beneficial effects in a mouse model for amyotrophic lateral sclerosis were not observed (Pedersen and Mattson 1999). The CR regimen also prevents age-associated declines in psychomotor and spatial memory tasks (Ingram et al. 1987) and loss of dendritic spines necessary for learning (Moroi-Fetters et al. 1989) and improves the brain's plasticity and ability for self-repair (Mattson 2000).

Why does CR exert these effects? Because CR delays reproduction and promotes survival in times of scarcity, it may have been evolutionarily adaptive during boom/bust cycles (Harrison 1989; Holliday 1989). Despite the plausibility of this reasoning, several challenges to the significance of CR studies in the laboratory have been made. Perhaps the restricted animals live longer simply because controls are overfed to the point of ill health. However, regimens in which animals are fed controlled amounts of food rather than ad libitum still show beneficial effects of low calories (Weindruch and Walford 1988). Another objection is that inbred strains of rodents are not representative of animals in the wild. For example, lab strains are selected for rapid reproduction and large litters (Miller et al. 1999). It has been argued that these animals may accordingly have shorter life spans than wild strains. By this reasoning, CR may simply correct a defect that has been created by domestication. However, the generality of CR in many different organisms, as mentioned above, supports the argument against this criticism.

Even though benefits of CR have been known for many years, the mechanism(s) of its action remains unclear. Its complexity lies in multiple effects including metabolic, neuroendocrine, and apoptotic changes, which vary in intensity and exhibit striking differences among specific organ systems. Several major models to explain CR exist, but none satisfactorily integrates all of CR's effects. In this review, we address the question of how CR might function to extend life span. We begin with a summary of several aging theories and classical views about the action of CR. Then we discuss how CR extends the life span in *Saccharomyces cerevisiae*. We extrapolate these findings from yeast to mammals and consider metabolic, neuroendocrine, and apoptotic shifts that may trigger longevity in the higher organisms. We conclude with a model of CR that integrates its effects on mammals.

Mechanisms of aging and classical views on how CR works

Evolutionary biologists have argued that aging is a consequence of the inability of natural selection to cull out undesirable characteristics in a postreproductive phase of life. In the wild, for example, rodents may rarely experience a postreproductive period because of early death, for example, by predation. This implies that aging is multifactorial with many mechanisms contributing to the decline. Nonetheless, ideas that one or a few specific mechanisms may lie at the heart of aging have gained currency. A leading theory is that aging is caused by

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cumulative oxidative damage generated by reactive oxygen species (ROS) produced during respiration (Harman 1988). Oxidative damage to DNA, RNA, protein, and lipids has indeed been demonstrated to occur with aging (Fraga et al. 1990; Stadtman 1992; Head et al. 2002; Liu et al. 2002). Some investigators argue that this damage may limit life span. For example, overexpression of the enzyme superoxide dismutase (SOD), which reduces ROS, extends life span in *Drosophila* (Parkes et al. 1998) and in stationary phase yeast cells (Longo et al. 1999).

How might CR slow down the generation of damage in cells? It has been documented that oxidative damage is reduced in CR animals (Lee and Yu 1990). If CR were to slow metabolism, the production of ROS would decrease as a simple consequence. However, studies measuring metabolic rate in CR animals give conflicting results. The weight of evidence in rodents indicates that metabolism, as measured by oxygen consumption normalized to the reduced body mass of the animal, does not slow down (McCarter et al. 1985). Because the balance of existing data does not support a long-term overall reduction in metabolic rate, more subtle explanations must be adduced. One possibility is that a more efficient transport of electrons through the respiratory chain might reduce the production of ROS and slow aging (Weindruch et al. 1986; Duffy et al. 1989, 1990). Another is that an increased ability to detoxify ROS slows oxidative damage in CR. The data relating CR to detoxification of ROS is again conflicting. On the one hand, organisms tend to be more resistant to an acute challenge by an exogenous oxidative stressor. For example, life-long CR seems to increase expression of SOD in rat liver (Semsei et al. 1989). On the other hand, in genetically altered strains of mice, there is no consistent correlation in the expression levels of SOD and life span (Hauck and Bartke 2000).

Another theory suggests that lack of protein turnover may cause aging. Multiple studies of aging organisms have shown accumulation of aberrant (e.g., oxidatively damaged) proteins and a reduction in protein turnover (Lavie et al. 1982; Gracy et al. 1985). CR may slow down accumulation of these potentially harmful abnormal proteins by speeding up protein turnover (Taylor et al. 1989; Sohal and Weindruch 1996). How can CR accomplish this? As the body runs out of fat during CR, it may trigger the degradation of proteins, thereby increasing their turnover. Indeed, the age-associated accumulation of oxidized proteins declines with CR (Aksenova et al. 1998; Dhahbi et al. 1999), and the activity of the liver 20S proteosome may increase during CR (Scrofano et al. 1998). Microarray study of mouse skeletal muscle also showed an increase in protein synthesis and degradation during CR (Lee et al. 1999). However, the elevated turnover during CR is not uniform; although some damaged proteins were degraded, others continued to accumulate (Scrofano et al. 1998). All told, the data suggest an increase in protein turnover during CR, but whether this change has an impact on the rate of aging is uncertain.

The covalent modification of proteins by derivatives of glucose has also been shown to increase with age (Masoro et al. 1989; Smith et al. 1994; Cefalu et al. 1995; Sell et al. 1996). These modified adducts in macromolecules, termed advanced glycation end products (AGE), have been linked to age-related pathologies (Lee and Cerami 1992). A reduction of AGE during CR has been demonstrated (Masoro et al. 1989; Cefalu et al. 1995). The blood profile of CR animals predicts this reduction, because both glucose and insulin levels are reduced in CR animals (Masoro et al. 1983, 1992). However, a lower percentage of AGE during CR does not clearly explain the multiple other effects that are known to occur. It is unlikely that a decrease in AGE is responsible for the long life span in CR, because AGE is one of many degenerative changes in aging.

The same logic argues against any model proposing a single effect of CR on a mechanical aspect of the aging process, including the antioxidation model above (Fig. 1A). As we discuss below, studies on CR in yeast suggest that the increase in life span is a regulated response to food deprivation. Indeed, a regulated response to CR may account for the plethora of effects required to slow the wholesale decline in aging (Fig. 1B).

Regulation of yeast replicative life span by CR

In budding yeast, mother cells divide asymmetrically, giving rise to a newly made daughter cell and an aging mother cell. The mother cell adopts phenotypes of aging, including an enlarged size and sterility, and senesces after ~20 divisions. This aging has been linked to the repeated rDNA genes, which encode the large and small subunits of ribosomal RNA (Sinclair and Guarente 1997). Aging mother cells accumulate extrachromosomal rDNA circles, and mutations that slow the generation of these circles extend the life span. However, this rDNA instability has not been observed in other organisms, and is evidently an idiosyncratic feature of yeast aging.

The SIR2 gene regulates the life span in yeast mother cells; mutations that inactivate SIR2 shorten the life span, and overexpression of SIR2 extends it (Kaeberlein et al. 1999). SIR2 functions to silence chromatin by deacetylating the histones in targeted regions of the yeast genome, including the rDNA. The silenced chro-

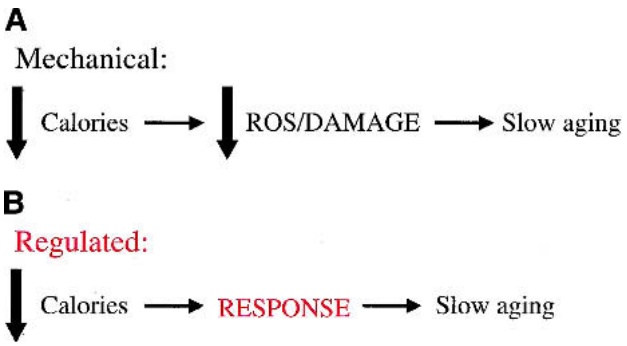

Figure 1. Two general mechanisms for how calorie restriction extends life span.

matin is structurally less accessible to RNA polymerase and to recombinational enzymes, thereby reducing gene expression and stabilizing repeated DNA. The Sir2p deacetylase is unusual because it requires NAD as a cosubstrate (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). NADH, NADP, and NADPH neither activate nor inhibit the enzyme (Imai et al. 2000).

CR can be imposed in yeast by reducing the glucose concentration in the media from the usual 2% to 0.5% (Fig. 2; Lin et al. 2000). Because cells continue to feed on yeast extract plus peptone, which are rich in amino acids, nucleotides, and vitamins, the growth rate remains rapid as glucose levels are lowered. Thus, the reduction in glucose from 2% to 0.5%, although modest, likely imposes a state of partial energy (ATP) limitation. Other dietary restriction protocols, which also limit amino acids and other nutrients (Jiang et al. 2000, 2002), drastically slow the growth rate and may make it more difficult to impose energy limitation.

Under our conditions of CR, mother cells divide ~30% more times. This additional life span does not occur in a sir2 mutant or in strains in which NAD synthesis is reduced (Lin et al. 2000). Therefore, the activity of Sir2p is required to deliver the long life span by CR, and indeed, the silencing activity of Sir2p was shown to increase in CR cells.

How does CR activate Sir2p in yeast? When the glucose levels in the media are lowered, yeast cells respond by shunting more of the carbon to the TCA cycle to generate ATP by respiration (Lin et al. 2002). This comes at the expense of fermentation, which is the preferred pathway of carbon use when glucose levels are high. This metabolic shift makes sense because cells harvest much more ATP by metabolizing the glucose to CO_2 in the TCA cycle than by fermenting it to ethanol.

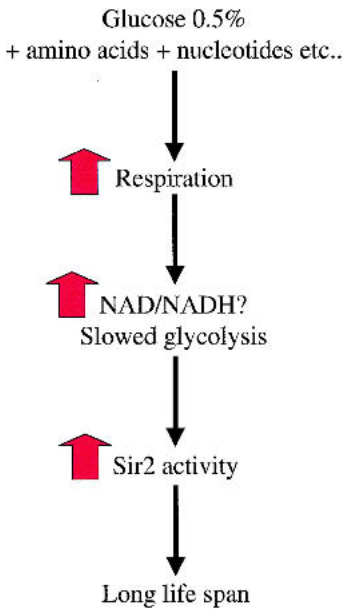

Figure 2. Calorie restriction triggers a regulatory response in yeast.

The shift toward respiration is necessary and sufficient to extend the life span in yeast. It is still not certain how this shift activates Sir2p to provide greater longevity. One possibility is that the activation of respiration converts more NADH to NAD and the resulting increase in the NAD/NADH ratio activates Sir2p. It has also been suggested that nicotinamide, which is generated during the deacetylation reaction and can inhibit Sir2p in vitro, is a negative regulator of Sir2p in vivo (Bitterman et al. 2002). There is still no direct evidence for either of these models. Another possibility is that the increase in respiration during CR slows glycolysis, and this metabolic change activates Sir2p. Any mechanism for this latter effect is at present unknown.

The important lesson from the yeast studies is that the extension in life span by CR is not a mechanical consequence of a reduction in ROS or AGE. Rather, the extension is a regulated response requiring SIR2 (Fig. 1). This regulation must involve a qualitative shift in metabolism that can be sensed by Sir2p. The deacetylase activity of the enzyme must then slow any degenerative processes that limit the life span.

Metabolic changes during CR in mammals

Studies suggest that there are two phases of CR in mammals, an adaptive period immediately after the regimen is imposed, and a steady state period, which can last the lifetime of the animal. During the adaptive phase, metabolism, as measured by oxygen consumption, declines (McCarter and McGee 1989). Glucose metabolism regulates secretion of insulin from the pancreatic β-cells. Interestingly, the β-cells in the pancreas that make insulin have been reported to sense glucose not by the production of ATP but by the conversion of NAD to NADH (Dukes et al. 1994; Eto et al. 1999; Antinozzi et al. 2002).

In the immediate response to low levels of glucose, the restricted animal quickly degrades glycogen stores upon the secretion of glucagon from the pancreas. When stored carbohydrates have been depleted, the animal starts to break down fats to compensate for the lack of glucose in the blood (Bertrand et al. 1980). As a result, loss of fat mass is one of the most striking phenotypic changes observed in calorically restricted animals (Barzilai and Gabriely 2001). The liver assumes a dominant role during CR by up-regulating the expression of enzymes involved in gluconeogenesis and down-regulating those in glycolysis (Dhahbi et al. 1999, 2001). Moreover, the liver makes ketones, such as acetoacetate, that result from the degradation of fat and proteins in the animal (Fig. 3).

After this adaptive period, the organism reaches a steady state in which ketones help meet energy needs of the brain. Thus, blood glucose falls precipitously during the adaptive period, and rises to a higher, but still below-normal level during the steady state (Greene et al. 2001). β-Cells sense the low glucose levels, and produce less insulin. The blood insulin level is lower than in control animals, and insulin sensitivity, that is, the efficacy of insulin in triggering glucose uptake by cells, is higher

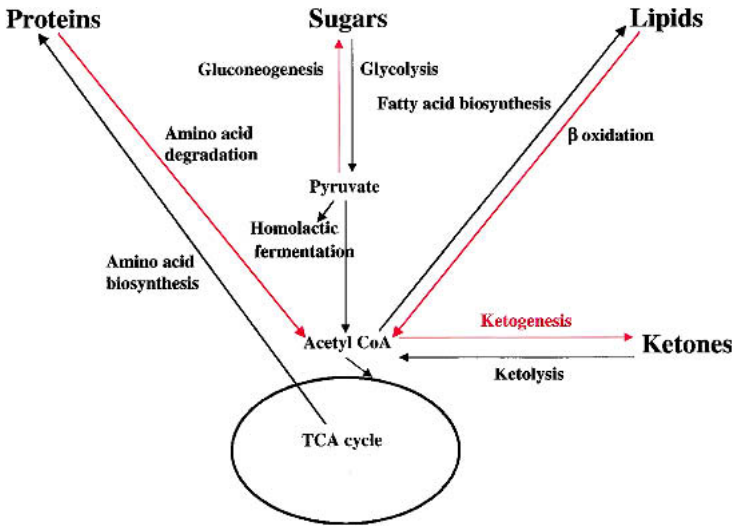

Figure 3. Metabolic pathways affected by calorie restriction in mammals. Red implies up-regulation, black implies down-regulation or no change.

(Reaven et al. 1983; Masoro et al. 1992; Cartee et al. 1994; Dhahbi et al. 2001).

Another metabolic alteration during CR in rodents, as well as in nonhuman primates, is a reduction in body temperature (Weindruch and Walford 1988; Duffy et al. 1990; Lane et al. 1996). This change may be due to an increase in the coupling of oxidative phosphorylation to ATP synthesis, perhaps by a reduction in levels of uncoupling proteins. These proteins span the inner membrane of the mitochondria and may allow proton leakage and thus hijack a fraction of the energy of electron transport to generate heat rather than ATP (Weindruch et al. 2001).

CR and the neuroendocrine system

The neuroendocrine system has long been thought to play a role in senescence (Landfield 1978; Finch et al. 1984; Sapolsky et al. 1986; Weindruch and Walford 1988). An attractive feature of this system is that it can coordinate effects on every tissue in the body (Nelson et al. 1995). Studies from *Caenorhabditis elegans* also provide strong evidence for the importance of hormones in aging, in particular for hormones involved in glucose metabolism. daf-2 is a worm ortholog to receptors for insulin and for its related peptide hormone, IGF-1 (Kimura et al. 1997). Mutant worms with reduced functional daf-2 (or several other components of the insulin/IGF-1 pathway, such as age-1 or pdk-1) live longer (Kenyon et al. 1993). Furthermore, the insulin/IGF-1 signaling pathway in worms is also responsible for triggering developmental arrest at the L2 larval stage in response to stress, such as the lack of food (Finch and Ruvkun 2001). These arrested L2 larvae, termed dauers, survive for long periods and can resume development to adulthood when conditions improve.

Many changes observed in the CR rodents trace directly or indirectly to changes in the neuroendocrine system. CR animals routinely have lower levels of the pituitary growth hormone (GH), thyroid stimulating hormone, IGF-1, and gonadotropins (Mobbs et al. 2001). Conversely, glucocorticoids, catecholamines, and glucagons increase (Klebanov et al. 1995; Mobbs et al. 2001). Mutations that affect expression of several hormones have also been shown to prolong life span. For example, Ames dwarf mice carry a loss-of-function mutation in the gene Pit^{dw} (Brown-Borg et al. 1996) that leads to abnormally low expression of GH (Sornson et al. 1996). Because GH normally turns on expression of IGF-1 in the liver, these mutant mice also have low levels of IGF-1 (Bartke et al. 1998; Coschigano et al. 2000). Ames dwarf mice live longer than wild type (Brown-Borg et al. 1996), whereas transgenic mice secreting large amounts of GH have a shortened life span (Laron 2002).

The synthesis and release of GH from the pituitary is controlled by the hypothalamus. This compartment of the brain releases hormones, such as growth-hormone-releasing hormone, that stimulate the release of GH from the pituitary. Interestingly, particular neurons in the hypothalamus may also stimulate the release of GH (Mobbs et al. 2001). The brain's ability to sense glucose is accomplished via a subset of hypothalamic neurons, which can sense the sugar and fire in response to threshold changes in its concentration. Amazingly, like the β-cells in the pancreas, these hypothalamic neurons sense glucose by the conversion of NAD to NADH (Yang et al. 1999). We speculate below that mammalian Sir2 proteins in the hypothalamus (and pancreas), because they are NAD-dependent deacetylases, sense CR and mediate the down-regulation of GH (and insulin).

Molecular analysis has the potential to strengthen the clues provided by the physiological and cellular studies above. Transcription profiling provides useful information in the brain. Expression of 6347 murine genes in 5- and 30-month-old mice, as well as CR versus control animals, was analyzed by microarrays (Lee et al. 2000). Several interesting patterns of gene expression were observed. First, genes reflecting oxidative stress and inflammation were induced in 30-month-old control mice. This induction did not occur in age-matched CR animals. Second, genes involved in protein synthesis were

the largest class reduced in expression during CR. A reduction in protein synthesis in the brain is not surprising, because the CR brain derives much of its energy from ketones, which are less energy-rich than glucose. Third, the expression of genes for growth and neurotrophic factors increased during CR. This includes genes encoding neuroplasticity factors such as neuroserpin. This finding may help explain the improved psychomotor performance observed in CR animals (Ingram et al. 1987).

Links between CR, aging, and apoptosis

Several recent studies suggest that apoptosis may limit mammalian life span. Mice with a targeted disruption in the p66shc gene exhibit a longer life span than wild-type animals (Migliaccio et al. 1999). Importantly, cells derived from the p66shc mice are resistant to DNA-damage-induced apoptosis in culture. Further, p66shc is one of the down-stream targets of the key regulator of damage-induced apoptosis, the tumor suppressor p53 (Trinei et al. 2002). In the cell culture studies, p66shc cells were resistant to oxidative stress or ionizing radiation, which both kill cells by the p53-dependent cell death pathway. This finding suggests that apoptosis may be a two-edged sword, providing critical tumor surveillance during the reproductive years, but contributing to organ dysfunction and aging in a postreproductive period.

A second finding may also implicate apoptosis in mammalian aging. The yeast SIR2 gene appears to promote survival in a wide range of organisms. In yeast this gene promotes long life span in mother cells, and is also crucial to the generation of the long-surviving, specialized cell type termed spores. In *C. elegans*, an organism that diverged from the yeast lineage a billion years ago, the SIR2 ortholog sir-2.1 also promotes long life in adult animals and regulates the formation of dauers during development (Tissenbaum and Guarente 2001). Recently, it has been shown that a cytoplasmic Sir2p homolog can promote survival in the protozoan parasite *Leishmania* by preventing apoptosis (Vergnes et al. 2002). The mammalian ortholog of SIR2, SIRT1, represses the activity of p53 and therefore down-regulates apoptosis (Luo et al. 2001; Vaziri et al. 2001). If the survival function of SIR2 genes observed in yeast, worms, and protozoans extends to mammals, apoptosis may thus be important in limiting mammalian life span.

Furthermore, a hyperactive allele of p53 has been described that confers enhanced tumor surveillance on transgenic mice (Tyner et al. 2002). Interestingly, these mice develop early organ degeneration and signs of premature aging. These phenotypes further support the idea that apoptosis may limit mammalian life span, because its enhancement apparently speeds up the aging process.

The above studies raise the possibility that any process extending mammalian life span would have to slow down apoptosis. However, in some organs with rapidly dividing cells, apoptosis actually increases during CR, for example, in the liver (James et al. 1998) and the gut (Holt et al. 1998). This increase, along with the known shrinkage of cells during CR (Birchenall-Sparks et al. 1985), may both contribute to the down-sizing of these organs in the restricted animal. The increased rate of apoptosis may minimize the risk of cancer during CR (James et al. 1998). The increase in apoptosis in these organs appears at odds with any central role for SIR2. However, it is possible that neuroendocrine changes are dominant in up-regulating apoptosis in this subset of organs.

The brain is the one organ that does not shrink during CR (Keenan et al. 1995; Weindruch and Sohal 1997). Is it possible that the link between apoptosis and life span discussed above is due to effects on neurons? Could p66shc KO mice live longer because neuronal death is slowed? It would be of interest to determine whether CR slows cell death of neurons. This may be difficult to visualize in animals, because apoptosis is transient and the number of apoptotic cells at any given time will be low. However, it may be possible to test whether interventions that slow aging, such as CR, result in less apoptosis when neuronal cells are harvested and cultured.

Model for CR effects on mammalian aging

We have described metabolic, hormonal, and other changes, which coalesce to slow aging and extend the life span of animals during CR. Several classical models for CR propose a mechanical basis for the slowing of aging and extension of life span. For example, the accumulation of damage by oxidation or glycation may be expected to slow down as a consequence of reducing calories in the diet. But experiments in yeast show that the added life span during CR is not a mechanical output of low calories, but a process that is highly regulated. In this organism, CR triggers a metabolic shift toward respiration that activates the regulator SIR2. Could the extension in mammalian life span by CR also be a regulated process?

We can model the chain of events that follows the imposition of CR in mammals (Fig. 4). We speculate that the altered physiology resulting from lower levels of calories induces primary changes in the neuroendocrine system. As mentioned above, glucose-sensing neurons in the hypothalamus, as well as β-cells in the pancreas, somehow recognize a slowing in the rate of conversion of NAD to NADH during CR. This metabolic change likely results in a reduction in the secretion of growth hormone from the pituitary and insulin from the pancreas. Low GH would in turn reduce levels of IGF-1 made by the liver. In addition to affecting IGF-1, the lowering of other pituitary hormones, the gonadotropins, slows reproductive capabilities of the animal.

We speculate that mammalian Sir2 proteins may play roles at two critical positions in the pathway of CR effects on aging. The first would occur during the sensing of CR, leading to changes in levels of hormones in the blood stream. Because Sir2 proteins are NAD-dependent deacetylases, they are well suited to this regulatory function and may play key roles in the pituitary and pancreas in sensing the conversion of NAD to NADH and resetting the levels of insulin and IGF-1 that are released. Such a mechanism would bespeak a conserved role of

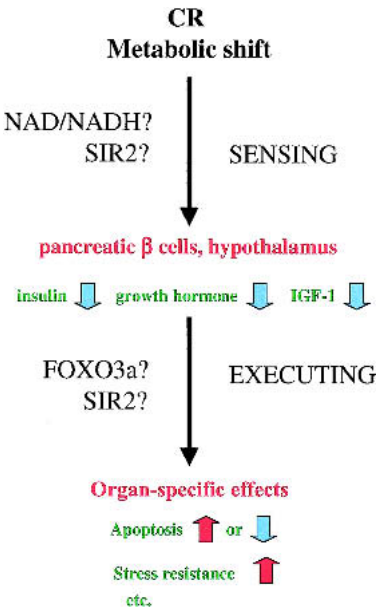

Figure 4. Model of how calorie restriction may extend life span in mammals. Effects occur at two levels: (1) sensing CR to adjust hormonal levels and (2) executing a slowing of aging on all organs. Roles for Sir2 genes are proposed at both levels, as discussed in the text.

Sir2 proteins as sensors of CR from yeast to mammals. The resulting endocrinological changes would allow the animal to mount a coordinated regulatory response to CR in different tissues.

Any hormonal changes must execute their effects by slowing aging in the animal. This execution phase is likely mediated, at least in part, by the insulin and IGF-1 signaling pathways in receptor-bearing cells. The precise effects of the hormone–receptor interaction may vary from organ to organ, because different cells bear different constellations of regulators. In general, however, longevity-promoting effects are expected to result from decreasing the insulin and IGF-1 pathways. In both *C. elegans* and mammals, these pathways impinge on transcription factors of the forkhead family. The worm factor is Daf-16 and the mammalian homologs are FOXO1–FOXO3. Decreased signaling by insulin/IGF-1 activates forkhead transcription factors, which in turn increase resistance to stress in mammalian cells (Nemoto and Finkel 2002; Tran et al. 2002) and worms (Guarente and Kenyon 2000). An increase in stress resistance is a hallmark of CR in a wide variety of organisms.

Changes that are not mediated by hormones may also be important. For example, metabolic changes on their own may directly slow aging in organs. In this regard, mammalian Sir2 proteins may play a pivotal role in some organs by recognizing the altered metabolism. If the metabolic shift during CR increases the activity of SIR2 in tissues with nondividing cells, it may directly slow apoptosis and age-dependent degeneration of organs such as the brain and perhaps the heart. Finally, metabolism-mediated changes in cells may synergize with changes in the levels of circulating insulin/IGF-1 hormones. In this regard, it is interesting that the worm sir-2.1 extends life span by down-regulating the insulin/IGF-1 responsive signaling pathway. It is tempting to speculate that mammalian Sir2 proteins play a second role during this execution phase of CR by modulating the insulin and IGF-1 signaling pathways in hormone-responsive cells.

Extension of life span by CR in mammals is a multidimensional phenomenon, which has ramifications ranging from endocrinology to metabolism to cell biology. In this review, we have discussed a regulatory model for how CR could extend life span in mammals. The studies in yeast imply that the extension of life span by CR is a regulated process. It is important therefore to consider regulatory mechanisms in any discussion of how CR slows aging in mammals. We have proposed one such model of how a coordinated global response to metabolic changes could work.

Acknowledgments

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