Development of Calorie Restriction Mimetics as a Prolongevity Strategy

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ABSTRACT: **By applying calorie restriction (CR) at 30–50% below *ad libitum* levels, studies in numerous species have reported increased life span, reduced incidence and delayed onset of age-related diseases, improved stress resistance, and decelerated functional decline. Whether this nutritional intervention is relevant to human aging remains to be determined; however, evidence emerging from CR studies in nonhuman primates suggests that response to CR in primates parallels that observed in rodents. To evaluate CR effects in humans, clinical trials have been initiated. Even if evidence could substantiate CR as an effective antiaging strategy for humans, application of this intervention would be problematic due to the degree and length of restriction required. To meet this challenge for potential application of CR, new research to create "caloric restriction mimetics" has emerged. This strategy focuses on identifying compounds that mimic CR effects by targeting metabolic and stress response pathways affected by CR, but without actually restricting caloric intake. Microarray studies show that gene expression profiles of key enzymes in glucose (energy) handling pathways are modified by CR. Drugs that inhibit glycolysis (2-deoxyglucose) or enhance insulin action (metformin) are being assessed as CR mimetics. Promising results have emerged from initial studies regarding physiological responses indicative of CR (reduced body temperature and plasma insulin) as well as protection against neurotoxicity, enhanced dopamine action, and upregulated brain-derived neurotrophic factor. Further life span analyses in addition to expanded toxicity studies must be completed to assess the potential of any CR mimetic, but this strategy now appears to offer a very promising and expanding research field.**

KEYWORDS: **metabolism; body temperature; stroke; MPTP; 2-deoxyglucose; metformin; insulin**

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CALORIE RESTRICTION

The most robust and reproducible prolongevity intervention in laboratory rats and mice is to reduce caloric intake by 30–50% below *ad libitum* (AL) levels over their adult life span. Evidence has emerged from hundreds of rodent studies that such calorie restriction (CR) regimens can significantly increase mean and maximum life span, reduce the incidence and age of onset of age-related diseases, increase resistance to numerous stressors and toxins, and maintain function later into life.[1–3] Although much less investigated in nonrodent species, the CR paradigm has been used to demonstrate its prolongevity effects in several other invertebrate species, including daphnia, nematodes, fruit flies, and spiders, and short-lived vertebrate species, including fish and reptiles.[2]

Recent studies of CR in rhesus monkeys have not yet generated definitive conclusions regarding effects on mortality and morbidity in a long-lived primate species, but it is clear that CR in monkeys produces physiological effects that parallel those observed in rodents.[4–6] Moreover, established risk factors for diabetes and heart disease are also reduced in monkeys on CR.[5,6] If the findings in these primate studies continue to produce evidence that aging can be retarded in a species closely related to humans, then the relevance of CR as an intervention in human aging will be strongly supported.

Abundant epidemiological data have been reported to demonstrate that caloric intake is related to the incidence of many chronic diseases, including cardiovascular disease, cancer, and diabetes, as well as neurodegenerative disorders.[7–12] However, experimental evidence that CR can retard aging processes in humans has not been established. A study of a small group of volunteers confined to Biosphere 2 confirmed that CR could be imposed for two years and would produce many of the physiological, hormonal, and morphological effects expected.[13] To further evaluate this feasibility of CR intervention in humans,[14] recent clinical studies of CR sponsored by the National Institute on Aging have been initiated at three sites—Washington University, Tufts University, and the Pennington Center at Louisiana State University. These studies will provide valuable information on physiological responses to CR over a short term (3–5 years), as well as greatly needed information on procedures to obtain and maintain compliance for such regimens.

Considering the epidemiological studies reporting an association between disease and caloric intake and assuming future results from the clinical trials under way will be positive, the foremost question will remain whether CR can alter the rate of aging in humans. If we can assume that the answer to the question is positive, then the overarching question is the following: even if CR was a well-established intervention to retard aging and age-related disease, would it be a practical antiaging prescription? Given the documented difficulty in Western culture to maintain low calorie diets for a prolonged period,[15] much less a major portion of the lifetime, the application of CR to humans could be problematic due to the severity and length of restriction required. Thus, we need to pose the following question: are there alternatives to CR that can provide its antiaging benefits without actually having to drastically reduce caloric intake?

CALORIE RESTRICTION MIMETICS

To address this question, we have developed a research program to assess interventions designed as "CR mimetics." The intent of this strategy is to produce the same prolongevity effects that CR provides, but without substantially reducing caloric intake or otherwise inhibiting food intake.[16–18] CR mimetics can be pharmaceuticals, nutraceuticals, hormones, diets, or even genetic manipulations that provide the benefits of CR without its dietary demands. In our consideration of this concept, we have steered away from other possible means of reducing caloric intake. For example, CR could be induced by restricting the digestive tract (e.g., stomach stapling) or it might be achieved by successful appetite suppression through pharmaceutical means. Instead, we have directed our efforts to interventions that target specific metabolic pathways involved in mediating the effects of CR. In effect, by activating protective mechanisms that are activated in CR, the organism could be "tricked" into a CR state when its actual caloric consumption had not been changed or changed to much less extent than conventional CR regimens would dictate.

If actual reduction in dietary calories is assumed to be the major factor driving the prolongevity effects of CR, then what would be the appropriate mechanism(s) to target for a CR mimetic? Regarding mechanisms of CR, several hypotheses have been proposed, including reducing oxidative stress,[19] controlling inflammatory responses,[20] and protecting against glycation of macromolecules.[21] Interventions that protected against these deleterious processes might have prolongevity effects. From our perspective, however, it would be more appropriate to target a broadly acting mechanism, such as enhancing stress responses to condition the organism to handle all these deleterious processes.

POSTULATED PROLONGEVITY MECHANISMS

One compelling hypothesis to explain the prolongevity effects of CR is that it induces an evolutionarily conserved stress response that conditions the organism toward enhanced stress protection.[22–26] Some investigators have invoked the concept of hormesis from toxicology, which implies that small doses of a toxin might have long-term beneficial consequences as a means of conditioning the organism toward enhanced stress responses.[25,27,28] From an evolutionary perspective, the disposable soma theory of aging proposes that, during times of low energy availability, the organism must shift away from energy investment in growth and reproductive processes to energy investment in somatic maintenance and repair.[29] The essential feature of this hypothesis is that it is adaptive for the organism under CR to use available means to protect itself and thus enhance its stress protective mechanisms. Hormonal evidence that CR induces stress is manifested in higher levels of glucocorticoids.[25] Increased stress responses of rodents on CR have been demonstrated in numerous paradigms.[25] Several studies assessing response to neurotoxins in rats and mice on various regimens of CR have shown increased protection.[30–33]

A well-studied example of the adaptive strategy of CR is the diapause observed in the nematode, *Caenorhabditis elegans*. When exposed to low energy environments, this organism converts to its dauer state in which development and reproduction are

arrested. Within the dauer state, this roundworm is more stress resistant and can markedly exceed the life span of its normal adult form.[34,35] Several key genes have been identified that appear to regulate conversion to the dauer form, among them age-1, daf-2, daf-16, daf-18, akt-1, and akt-2.[36] This signal transduction pathway appears to be homologous to an insulin/insulin growth factor-1 (IGF-1) pathway in mammals.[34,35,37,38] Selected mutations of genes regulating this pathway, for example, daf-2, can reduce signaling such that the worm does not transform to the dauer form, but does have markedly extended life span associated with increased stress protection.[37,38] Mammalian models of reduced signaling through the insulin/IGF-1 pathways have also produced evidence of prolongevity effects.[39] Specifically, knockout mice for the IGF-1 receptor exhibit increased life span and increased resistance to oxidative stress. There appears to be little other phenotypic effects observed in this mouse as their energy metabolism, nutrient uptake, physical activity, fertility, and reproduction do not differ substantially from normal littermates. Similar prolongevity findings have been found in transgenic rats[40] and mutant dwarf mice[41] in which the growth hormone/IFG-1 pathway has been affected, but which differ along other phenotypic parameters from their normal controls.

2-DEOXYGLUCOSE

Studies in invertebrate and vertebrate models of prolongevity point to a logical strategy of inducing stress resistance by manipulating systems involved in energy sensing, regulation, and metabolism. Thus, as an initial target for our CR mimetic strategy, we focused on glucose metabolism. We hypothesized that inhibition of glycolytic pathways could mimic CR without altering food intake. As an initial approach,[16] rats and mice were fed a diet containing 2-deoxy-D-glucose (2DG), which inhibits the enzyme, phosphohexose isomerase, and thus reduces glycolytic processing. Previous studies revealed that 2DG injections could inhibit tumor growth[42] and produce torpor[43] and increase glucocorticoids,[44] all of which paralleled effects of CR.

In the initial study,[16] young male Fischer-344 (F344) rats were fed diets supplemented (by weight) with 0.2%, 0.4%, or 0.6% 2DG, which was approximately 100–150, 250–300, or 400–450 mg/kg, respectively. The high dose proved to be toxic as a couple of deaths were observed after a few weeks, so this group was then fed the 0.6% diet every other week, which appeared to be generally well tolerated. Acute toxicity would be expected at some 2DG dose because of insufficient cellular energy due to an intolerably high degree of glycolysis inhibition. The major end points of the study were physiological. As observed in FIGURES 1 and 2, the 2DG diet reduced plasma insulin and body temperature at the 0.4% and 0.6% concentrations, without significant reduction in plasma glucose. Although all doses initially reduced food intake and body weight, the 0.2% and 0.4% groups caught up to controls after a few weeks so that, by the end of the study at 6 months, they did not differ significantly from controls. Thus, because two major biomarkers of CR had been affected in this study, specifically reduced insulin and body temperature, we concluded that 2DG was a promising candidate as a CR mimetic.

The validity of these biomarkers of CR was further confirmed in a recent analysis of survival data in human males derived from the Baltimore Longitudinal Study of

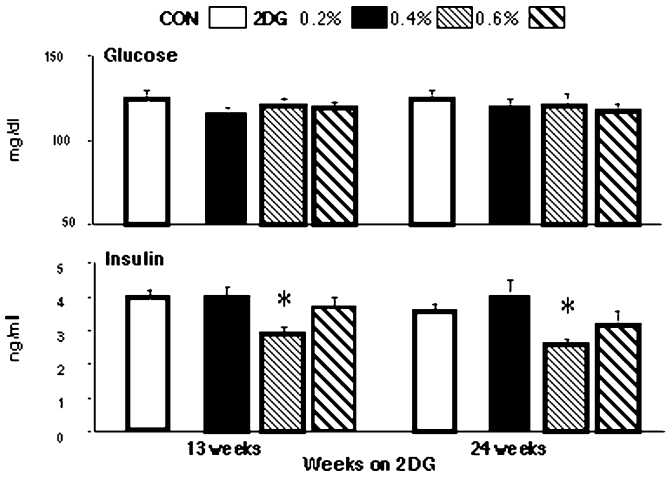

FIGURE 1. Mean (SEM) plasma concentrations of glucose and insulin in male F344 rats fed control (CON) diet or diet supplemented with 2-deoxyglucose (2DG) in three concentrations. *Significantly different from CON group.

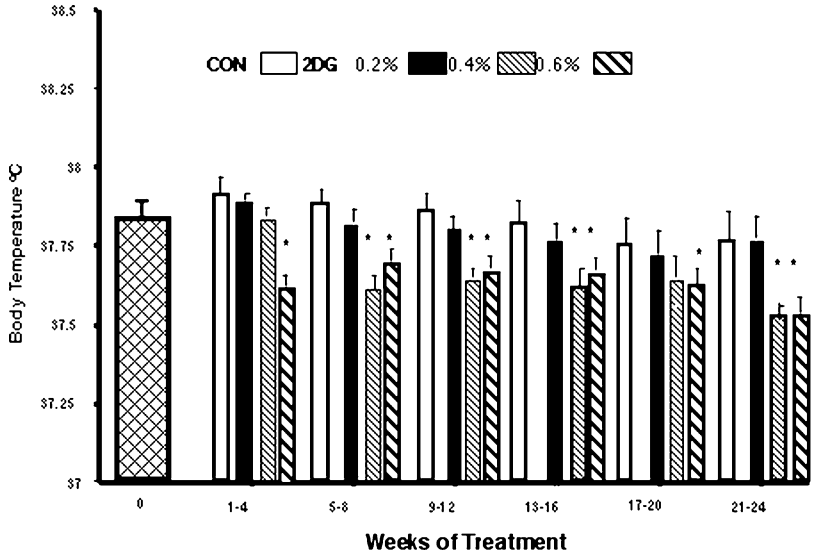

FIGURE 2. Mean (SEM) body temperature recorded in male F344 rats fed control (CON) diet or diet supplemented with 2-deoxyglucose (2DG) in three concentrations. *Significantly different from CON group.

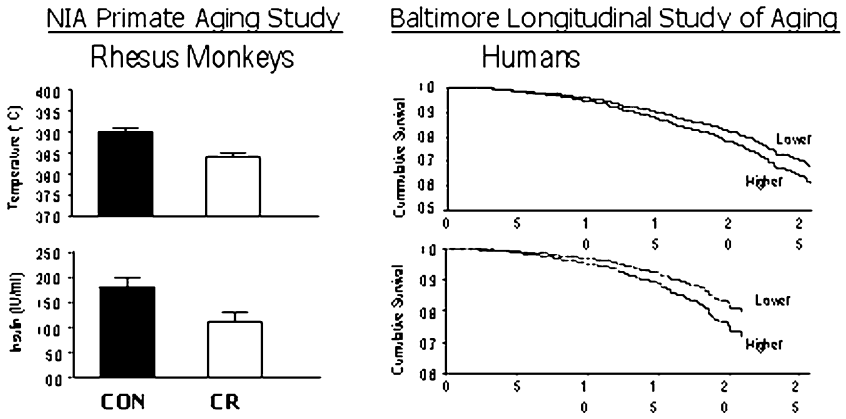

FIGURE 3. (*Left*) Mean (SEM) plasma insulin and body temperature of male rhesus monkeys on control (CON) or calorie restricted (CR) diets, and (*right*) survival probability curves of healthy males enrolled in the Baltimore Longitudinal Study of Aging based on whether in the top or bottom half of the distributions for body temperature and plasma insulin.

Aging (BLSA). Specifically, as presented in FIGURE 3, we examined the probability of survival in a healthy sample of men and found that those with the lowest temperature and plasma insulin levels had the lowest mortality risk.[45]

In vitro analysis of 2DG effects confirmed the ability of the compound to enhance protection against a number of stressors. For example, 2DG protected fetal hippocampal neurons against glutamate excitotoxicity.[46] Upregulation of heat shock protein-70 (HSP-70) and glutamate responsive protein-78 (GRP-78) was observed in the 2DG-treated cultures to confirm that stress pathways induced in CR rats could be duplicated. When rats were given 2DG injections for seven days, their cortical synaptosomes showed greater protection against iron and amyloid-β-peptide, and HSP-70 and GRP-78 were increased compared to control synaptosome preparations.[47] In an *in vivo* model of focal ischemia using middle cerebral artery occlusion-reperfusion, 2DG feeding attenuated damage similar to the effects of CR.[48] Mice injected with the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), exhibit marked motor impairments related to depletion of dopamine (DA) resulting from neuronal loss in the substantia nigra; however, 2DG injections provided greater protection and faster behavioral recovery.[49] Again, the stress proteins, HSP-70 and GRP-78, were increased in DA-rich brain regions in 2DG fed mice. A recent long-term study in rats comparing 2DG feeding to CR also noted many similarities.[50] Compared to AL controls, serum glucose and insulin were reduced in both CR and 2DG fed groups. Body temperature was also consistently lower in CR rats, but not so consistently lower in 2DG fed rats. However, heart rate and blood pressure were markedly decreased in both groups. In this study, all the physiological effects of CR, except body temperature, were reproduced in 2DG fed rats, for which food intake and body weight were similar to controls.

Given evidence of such robust effects of this compound, additional studies have addressed how the stress responses could be mediated. Of particular interest was how the brain might be involved. Recent studies have indicated the involvement of a neurotrophic factor, brain-derived neurotrophic factor (BDNF), which can be neuroprotective.[51] BDNF is also involved in enhancing DA neurotransmission. Past studies have demonstrated that short-term CR (e.g., 2 weeks) can increase DA-related locomotor responses.[52] Specifically, when challenged with the DA agonist, amphetamine, rats show hyperactivity, and this response is enhanced when rats are on CR. In recent studies, we have found that rats on CR for 4 months have enhanced locomotor response to amphetamine, and this response is also observed in rats fed 2DG.[53] Previously, it has been noted that CR can attenuate the age-related loss of striatal dopamine D2 receptors.[54] It would be interesting to determine if long-term 2DG feeding could produce similar results.

A variety of findings revealed a highly favorable profile for 2DG as a CR mimetic. What remained to be accomplished then was a long-term study to evaluate its effects on mortality and morbidity. To this end, we initiated a study of 2DG feeding in 6-month-old male F344 rats. We used the dose of 0.4%, which proved effective in the previous study, and a dose of 0.25%. Unfortunately, after about 2 months on the diet, deaths began to occur in the higher dose group, and these accelerated with time. A primary cause of death was congestive heart failure. In preliminary pathological analyses, we have found that rats on both 2DG doses had enlarged hearts with a marked vacuolization. We are currently completing this long-term study, but the results do not appear encouraging regarding this possible life-threatening pathology observed in 2DG fed rats. What this line of investigation demonstrates is that a useful screen for CR mimetics can be established, but the long-term studies must still be undertaken to prove the prolongevity effects of a candidate mimetic.

OTHER CALORIE RESTRICTION MIMETIC CANDIDATES

The number of possible CR mimetics would comprise a very long list. In addition to inhibiting glucose metabolism, another possible pathway to manipulate is insulin signaling. Again, many candidates could be suggested, but we have recently focused on the biguanides, specifically metformin. Metformin acts to reduce liver glucose production as well as acts as an insulin sensitizer. Known as glucophage, it is a highly prescribed medication in the treatment of type 2 diabetes.[55] Although we had not conducted much research with this compound, we were aware of previous positive life span studies using a related compound, phenformin, in mice.[56] In a recent *in vitro* study, phenformin was shown to increase resistance to glutamate toxicity in primary neuronal cultures.[57] Recently, we have undertaken a survival study in male F344 rats fed metformin (300 mg/kg). Preliminary findings indicate that this dose can reduce body temperature and plasma insulin, but without significant effects on body weight or food intake, similar to findings with 2DG.

Another inhibitor of glycolysis, iodoacetate acid (IAA), has also shown some promise. Pretreatment of fetal hippocampal neurons with IAA protected them against various stresses, including glutamate, iron, and trophic factor withdrawal, and upregulated heat shock proteins, HSP-70 and HSP-90.[58] Other possible candidates as CR mimetics could be found among the thiazolidinediones, which are agonists for

peroxisome proliferator activated receptors (PPARs). Recently, in diabetic monkeys, a PPARδ agonist markedly improved the lipid and insulin profiles,[59] and these compounds are now being assessed in several clinical trials. Pharmacological inhibition of IGF-1 signaling has also been suggested as a logical candidate for CR mimetics.[8]

DIET REGIMEN

As another approach to developing CR mimetics, we have been conducting studies of other regimens that might not require a severe degree of CR. A major tenet of the CR literature is that reduction in calories is required to achieve the age-retarding benefits of this intervention.[1–3] Because few investigators have compared different regimens of CR, such as feeding every other day versus limited daily feeding, we have begun such comparative studies. The major hypothesis being evaluated is that it is an adaptation to periods of food deprivation, rather than CR per se, that provides the protective effect. Moreover, we are interested in determining the role of the central nervous system as the initiator and mediator of these responses.

Although we had conducted many previous studies showing that intermittent feeding (IF), specifically feeding every other day (EOD), was an effective intervention for extending life span and retarding aging processes in rats and mice,[60–62] no studies had directly compared IF to CR imposed every day, that is, limited daily feeding (LDF). In a recent study,[63] IF in male C57BL/6J was found to be equally effective as LDF (40%) in providing protection against neurotoxicity induced by hippocampal injections of kainic acid (KA). FIGURE 4 shows that neuronal loss was

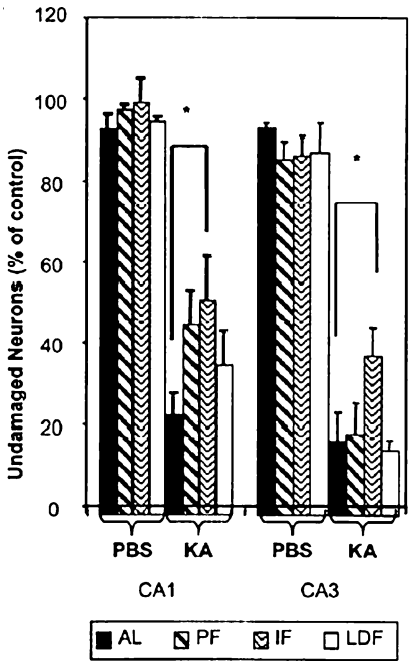

FIGURE 4. Percent reduction from controls in number of undamaged neurons in CA1 and CA3 hippocampal regions in response to injections of saline (PBS) or kainic acid (KA) in mice fed *ad libitum* (AL), intermittently fed (IF), pair-fed (PF) to the IF group, or fed 40% less daily (LDF). *Significantly different from AL group.

attenuated in both diet groups compared to KA-induced damage in AL fed controls. Indeed, in the CA3 region of the hippocampus, IF provided greater protection compared to LDF. The comparison between IF and LDF took on major significance when it was noted that the levels of restriction and body weight loss were modest in IF mice (~10%) compared to AL, yet insulin and glucose levels as well as neuroprotection were generally equivalent to the 40% LDF group. An interesting difference between IF and LDF was that serum IGF-1 levels were reduced as expected in LDF, but elevated in IF, whereas ketone bodies were increased in sera of IF and reduced in LDF mice compared to AL controls. Results of this experiment have led us to consider further that the regimen of CR, rather than the level of CR, might be the major factor for inducing the beneficial responses observed.

FUTURE DIRECTIONS

Because the number of candidate CR mimetics could be large, further development of CR mimetics would be accelerated by establishing a battery of assays to screen candidates. We have already mentioned the measurement of body temperature and plasma insulin levels as two important physiological screens. Examination of other hormonal responses, including glucocorticoids and thyroid hormones, as well as measurement of lipokines, such as leptin and adiponectin, could be added to the screen. Effects on tumor growth in experimental models could also make a highly relevant contribution to the screen. Other investigators have advocated the use of DNA microarrays to determine if a candidate CR mimetic produced, in a particular tissue, a pattern of gene expression that was highly similar to that produced by CR.[64] Many of the responses to CR, both genetic and physiological, are likely to be incidental to the prolongevity effect. The use of arrays in a comparison of the two different feeding paradigms, IF and LDF, offers a tool for eliminating many of the incidental effects and emphasizing those that are causally related to prolongevity.

The recent development in our laboratory of an *in vitro* model of CR could also be a highly useful component in a screening process for CR mimetics.[65] Specifically, a candidate compound could be provided to rats and mice, blood could be withdrawn, and subsequently extracted serum could be evaluated in a stress challenge. Serum derived from CR rats and monkeys has been shown to protect cells in culture from heat stress and oxidative stress provided by exposure to hydrogen peroxide. Thus, it stands to reason that serum derived from rats or mice treated with a candidate CR mimetic should afford cells grown *in vitro* greater protection against various stresses.

Prior to this *in vivo* screen, compounds could also be tested *in vitro* regarding their ability to protect cells against various stressors. This was the approach taken to evaluate phenformin and IAA as CR mimetics.[57,58] Even with a screen in place, any candidate CR mimetic would still need to be validated ultimately by demonstrating its beneficial effects on mortality and morbidity in a relevant animal model. Our experience in evaluating 2DG has shown the absolute necessity of such demonstration.

We believe that the concept of CR mimetics has opened up many new possibilities as a prolongevity strategy. We anticipate that many new approaches will be taken and many new candidates identified in many different laboratories as this strategy becomes increasingly popular and research directed toward this concept accelerates.

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