BMP REF 4

Spindler, Stephen R. (2003) Caloric Restriction, Longevity and the Search for Authentic Anti-Aging Drugs, In: Anti-Aging Therapy for Plastic Surgery, Kinney B. & Carraway J. (Eds.), Quality Medical Publishing, Inc., St. Louis. *In Press.*

STEPHEN R. SPINDLER

CALORIC RESTRICTION, LONGEVITY AND THE SEARCH FOR AUTHENTIC ANTI-AGING DRUGS

Department of Biochemistry, University of California, Riverside, CA 92521, U.S.A.; Biomarker Pharmaceuticals, Inc., Campbell, CA, U.S.A. E-mail: spindler@mail.ucr.edu; Voice: 909-787-3597; Fax 909-787-4434

1. INTRODUCTION

When asked "If you could live forever, would you and why?" as a part of the 1994 Miss USA contest, Miss Alabama is reported to have answered, "I would not live forever, because we should not live forever, because, if we were supposed to live forever, then we would live forever, but we cannot live forever, which is why I would not live forever". As beautifully circular, and distressingly illogical as her statement is, she is not alone. This view was somewhat more eloquently, but no less illogically expressed by Dr. Leon Kass, President Bush's leading scientific advisor. He is quoted as saying, "Confronted with the growing moral challenges posed by biomedical technology, let us resist the siren song of the conquest of aging and death". In other words, the ethical dilemmas associated with today's technologies are so challenging to his moral sensibilities that we should abandon attempts to understand aging, slow its ravages, and prevent death. This is an extremely chilling point of view for a physician to take. But, despite what Miss Alabama and Dr. Kass may think, it is not the siren singing, but instead it is the best of what makes us human.

Humankind has been successfully extending our average lifespans for at least the past two centuries. The ancient Romans left actuarial records and graveyards that have been excavated and studied. If you lived in Rome 2000 years ago, by the time you reached 25 years of age, about half the people born the same year as you were already dead. In fact, the shape of this human survival curve in Rome was similar to that of mice or spiders in the field, and drinking glasses in a restaurant (Fig. 1). Only the scale on the abscissa need be changed. Infectious disease, accidents, poor housing, and an unreliable and poor quality food supply more often than not led to brief, sickly, and brutal lives.

During the next 1800 years, average lifespan did not change from that of ancient Rome. By 1796, it stood at about 24 years. But, by 1900, advances in science and technology had created the industrial revolution, and the rise of the middle class in Europe and the USA. This resulted in improved sanitation, a higher quality and more abundant food supply, and better shelter. In response, lifespan doubled to about 60 years of age. Today, the average lifespan world wide has risen to 63 years. However, in industrialized countries, the increase in lifespan is even more dramatic. In the USA, life-expectancy has jumped to 77 years (72 for men and 79 for women), while in Japan, the world leader, the average lifespan is 80 years.

By the 1960s, the shape of the human survival curve in developing countries had begun to resemble that of mice in a vivarium (Fig. 1). This shape shows that we are closing in on the theoretical maximum lifespan of our species. Maximum lifespan is usually defined as the lifespan of the longest lived members of a species. For humans, this maximum lifespan is estimated to be around 120 years of age. We know of one human, Mme. Jeanne Calment, who was documented to have lived to be 122 years old.

Almost all of the spending for health-related research by the National Institutes of Health and the National Science Foundation, and all of the spending by state governments, private foundations and industries, and all of the health care spending on doctors, hospitals and pharmaceuticals are focused on moving our human lifespan the relatively small distance from our present survival curve to our ideal survival curve (Fig. 1). It has been estimated that if we conquer all the diseases which kill humans, including cancer and cardiovascular disease, which are by far the major killers in industrialized societies, we will obtain the theoretical maximum lifespan of

our species. But, lifespan will only be extended by another 15 years from what it is now (1). This points out the dilemma we face. No matter what we do about the diseases that kill us, we will eventually (if we are lucky enough to live that long) hit the wall of our maximum lifespan, which looms out there somewhere at around 120 years of age.

2. CALORIC RESTRICTION

However, scientists have known since the 1930's that a simple dietary treatment termed "caloric restriction", or CR, was capable of extending the maximum lifespan of a species by about 40% (Fig. 2). This effect of CR overcomes the boundary set by the theoretical maximum lifespan of a species in the absence of all diseases. It does something new, it not only delays the onset of almost all diseases, it also moves the lifespan curve to the right, creating a new maximum lifespan. Until recently, CR was the only effective method of doing this. This same result was obtained in almost every laboratory animal tested. At the same time, CR was found to reduce the incidence and severity of most age-associated diseases (2). These diseases include cancer and cardiovascular disease. A number of studies suggest that CR diets might work on human beings (discussed below).

The ability of CR to slow aging and extend maximum lifespan in mammals was first discovered by a Cornell University nutritionist, Clive M. McCay (2). McCay found that feeding rats smaller amounts of a nutritious diet led to longer-lived, healthier rats. His longest-lived CR rat lived for 48 months, while his longest lived fully-fed rat died at only 30 months of age. In later experiments at the Institute For Cancer Research in Philadelphia, Morris Ross was able to keep CR rats alive for more than 59 months (3). In the intervening years, the effects of CR have been reproduced hundreds of times in laboratories all over the world (4).

CR appears to slow the underlying rate of the aging, not just prevent or ameliorate the diseases of aging, although it also has these effects. Because CR slows the rate of aging, it shifts the survival curve to the right rather than simply moving it closer to the maximum theoretical survival curve for the species (Fig. 2). The effects of CR are dose dependent. The greater the degree of CR, the greater its effects, provided malnutrition is avoided (Fig. 2). By compiling the results of 24 published CR survival studies, Merry found that in rodents the duration of CR was directly proportional to the increase in survival (5). Using the same approach, he also demonstrated that in rodents, the greater the degree of CR, the greater the increase in lifespan. But, importantly for those of us who try to limit our caloric intake, just 10% underfeeding produces an appreciable increase in both average and maximum lifespan (6).

3. IS THE COMPOSITION OF DIET IMPORTANT?

Among possible dietary manipulations reported in the literature, including the restriction of fat, protein, carbohydrate, or minerals do not increase maximum lifespan unless the number of calories consumed is reduced (7). Further, there are no reproducible examples in the literature where maximum lifespan is extended by vitamin supplementation, administration of antioxidants, or variations in the type of dietary fat, carbohydrate or protein in the diet. In some studies, the lifespan of short-lived strains of mice and rats does increase with dietary manipulations other than CR. These effects probably arose by influencing disease susceptibility or progression rather than aging itself. Where tested, CR gives by far the most impressive lifespan extension and disease amelioration, especially in short-lived rodent strains.

4. PHYSIOLOGICAL EFFECTS OF CR IN ANIMALS

CR preserves or enhances the immune responsiveness in old animals, reduces the incidence and severity of age-related diseases, and delays or prevents the onset of cancer, diabetes, and heart disease (4, 6; 8). Thus, animals live longer and healthier lives. CR increases insulin sensitivity and glucose disposal, and resists the age-associated hyperinsulinemia which frequently develops with aging in both rodents, non-human primates and humans (9-13). CR does not prolong the period of "accelerated mortality" near the end of life, where animals get sick with age-related diseases such as cancer and diabetes. Instead, it prolongs the healthy period and postpones the period of accelerated mortality until near the end of a much longer life.

The effects of CR are robust. They appear to be conserved in dogs, rodents, fish, nematodes, spiders, flies, and protozoa (4, 14-16). However, the possibility that CR will produce similar effects in humans and other primates is still open to debate. There are a few who argue that it is unlikely that the maximum lifespan of humans can be extended by any technique, including CR (17). However, many gerontologists think that the evidence does not support such pessimism. A growing number of studies support the idea that CR does extend the lifespan of non-human primates, and perhaps that of humans as well.

5. CR IN PRIMATES

CR produces changes in the physiology of nonhuman primates which are strikingly similar to those produced in rodents (13, 18). Three major studies have examined the role of CR in nonhuman primates (19-21). A first report of reduced mortality in CR rhesus monkeys was presented in April of 2002 (22). At the NIA and University of Wisconsin, rhesus monkeys are fed 30% less than ad libitum (the amount they eat when given free access to food) controls. Preliminary data from the NIA study, which has been underway for 15 years, suggests that CR is reducing morbidity and mortality among the rhesus monkeys (13). Data from two different CR monkey groups at the NIA support a trend toward reduced cardiovascular disease, diabetes, neoplastic disease, and liver failure as causes of mortality (18). CR also reduces oxidative damage in monkey skeletal muscle (20). Life-long CR reduces body fat and trunk to leg fat ratio, suggesting a lower risk for cardiovascular disease (18). In primates, insulin and glucose profiles are improved (13). CR monkeys develop diabetes much later and with lower incidence than ad libitum fed monkeys (11). CR monkeys have decreased plasma triglyceride levels and increased high density lipoprotein levels, suggesting less risk of cardiovascular disease (13, 23, 24).

Several years ago, the NIA primate facility initiated a study in which young and old, male and female rhesus monkeys were shifted gradually from their regular ad libitum diet to a diet reduced by 30% in calories. We term such studies short-term CR (ST-CR) studies. Results from this study indicate that ST-CR rapidly improves disease-related markers associated with aging in old, male rhesus monkeys (25). Our unpublished studies using rhesus tissue biopsies from these monkeys indicate that ST-CR also produces changes in microarray profiles and metabolic enzyme gene expression and activities similar to many of the effects of CR in mice.

6. DOES CR WORK IN HUMANS?

The limited physiological evidence available for humans suggests that CR produces effects which are very similar to those found in CR rodents and monkeys (26-28). The difficulties of conducting long-term human CR studies have prevented many human CR studies from being done. However, physiological biomarkers associated with lifespan extension in CR rodents and monkeys also are associated with enhanced lifespan in humans (29). Lower body temperature and reduced plasma insulin levels are two of the most robust biomarkers of CR in rodents and rhesus monkeys. CR also has been found to slow the normal decline in serum dehydroepiandrosterone sulfate in rodents and rhesus monkeys. Men with lower body temperature and blood insulin levels, and higher blood DHEAS levels live longer than men who do not. These results suggest that the same molecular-genetic processes which lead to lifespan extension in CR animals may also extend lifespan in humans. Human genetic variability may lead to activation of some of these lifespan extending processes in a few fortunate humans.

Despite a lack of good data, natural episodes of human CR are distressingly common in some parts of the world. However, almost without exception these populations are exposed to poor quality CR diets. Poor quality diets are especially stressful for humans, since we have lost the ability to synthesize some nutrients such as vitamin C, that are produced endogenously by animals such as rodents, who live near the bottom of the food chain. However, many of the effects of CR in humans mimic the physiological effects in mice. Human CR is associated with short stature and late reproductive maturation, lower baseline gonadal steroid production in adults, suppressed ovarian function, impaired lactation, reduced fecundity, weakened immune function, and lower basal metabolic rate, pulse rate, body temperature and blood pressure (30-36).

There are only a few studies of human health and longevity using a high-quality, CR diet. The studies of Kagawa found that the total energy consumed by Okinawan school children was 62% of the recommended intake for Japan. Adults consumed 20% fewer calories than the national average. The diet was high quality, consisting largely of vegetables, rice and some fish. Death rates from cerebral vascular disease were reduced to 59%, malignancy to 69% and heart disease to 59% of the rest of Japan. The mortality rate for 60-64 years olds living on Okinawa Island in the 1970s was half the rate found elsewhere in Japan (37). The incidence of centenarians on the island was two to forty times greater than that of other Japanese communities. These excellent morbidity and mortality statistics are most likely associated with the Okinawan diet. Okinawans who emigrate to the USA and begin to consume a more typical Western diet have mortality and morbidity rates similar to those of other USA inhabitants. These data are consistent with the hypothesis that CR increases lifespan and reduces the incidence and severity of age-related diseases in humans.

There are two other studies which directly related to the question of CR in humans. In the first of these, 60 volunteers received a dietary regimen averaging 1500 kcal/day for 3 years, versus 2300 kcal/day in the controls (38). The CR group was fed 2300 kcal/day every other day, and one liter of milk and 500 g of fruit on the other day. Reanalysis of these data indicated that CR resulted in lower rates of admission to the infirmary (123 versus 219 days), and a nonsignificant lowering of death rates (6 versus 13) (39).

However, from the number of calories fed to the CR group, it seems likely that they crossed over the line into starvation during the study.

A second human CR study, this one serendipitous, was conducted by Roy Walford on eight healthy nonobese humans on a low-calorie (1750-2100 kcal/day), nutrient-dense diet. The subjects were sealed for two years inside Biosphere 2, a huge, closed ecological space near Tucson, AZ (26, 27, 40). Longitudinal studies of 50 variables were measured in these subjects before, during and after confinement using a Bayesian statistical analysis (40). These data show that physiologic, hematologic, hormonal, and biochemical changes in the subjects while on the CR diet resemble those of rodents and monkeys on CR diets. Despite the dramatic restriction in calories and the marked weight loss, all crew members remained in excellent health and sustained a high level of physical and mental activity throughout the entire 2 years of confinement.

7. WHY DO THE EFFECTS OF CR EXIST?

A striking example of the evolutionary conservation of the response to CR is a study by Austad, conducted using bowl and doily spiders (Fig. 3). He compared the longevity of the spiders in the field to that of captive spiders fed different numbers of fruit flies per week. Adult female spiders lived about 8 days in the field. However, in the protected environment of the laboratory, the mean adult lifespan was 42.3, 63.9, and 81.3 days when they were fed five, three, and one flies per day. This study illustrates that even for spiders, the powerful mechanisms of CR operate on lifespan.

When a trait is strongly conserved between species throughout the phylogenetic scale, we can be confident that the trait is important to the successful reproduction or survival of individuals of a species. Usually, similar molecular mechanisms in all species are responsible for highly conserved traits. When natural selection finds a molecular mechanism that works, it tends to be evolutionarily conserved. The conservation of the insulin/insulin-like growth factor pathways in determining the lifespan of worms, flies, and mice which is discussed below is a good example of this type of conservation.

For this reason, we think that CR is an ancient and important mechanism which appeared early in the evolution of eucaryotes. The most widely accepted hypothesis proposes that CR allows younger individuals to age more slowly, and therefore delays the onset of puberty and reproduction in the young during periods when food is scarce (41). In mature animals, CR causes a decline in reproductive behavior and fertility, thereby limiting the number of offspring during times of scarcity. This prevents them from directing the few nutritional resources which are available into reproduction. Instead, they can spend that energy on metabolic processes and behaviors which enhance survival, such as finding more food (42, 43). Then, when food becomes more available, in the spring for example, the animals eat more calories, the young rapidly progress to puberty and the older animals begin reproduction. In this way the number of a species can increase rapidly, before they are overwhelmed by an increase in the number of their predators. We know that CR has these kinds of effects in worms, flies, mice, monkeys, and at least to some degree, in humans.

Some have proposed that CR animals live longer because scientists overfeed their control animals, making them ill with too much food. However, this does not seem to be the case. Animals fed only 10% less food than ad libitum show the beneficial effects of CR (4). In fact, in most published rodent studies, the increase in lifespan obtained with CR is proportional with respect to the reduction in calories (5). The most parsimonious interpretation of these results is that CR extends lifespan, provided starvation is avoided. Some have argued that CR is not representative of animals in the wild because laboratory animals may have shorter lifespans than wild strains (43). However, the widespread phylogenetic prevalence of CR argues against this idea.

8. CAN EXERCISE MIMIC THE EFFECTS OF CR?

A common question is whether exercise can reproduce the effects of CR on lifespan. Exercise would seem to be a way to reproduce the effects of CR. Exercise does reproduce some effects of CR. Exercised animals are smaller and have less fat than sedentary animals fed the same number of calories. However, exercise does not extend maximum lifespan. Holloszy and his colleagues found that young rats, of about 4 months of age will run voluntarily almost 8000 meters per day if a running wheel is placed in their cages. As they begin to age, their running distances decline rapidly. But, if their food intake is reduced to 8% less than ad libitum consumption, their running behavior declines much more slowly (44). They will continue to run into extreme old age. These runners are much leaner and smaller overall than sedentary control rats fed the same number of calories as the runners.

If the effects of CR on lifespan result from its ability to retard growth, prevent excess body fat accumulation, and reduce dietary energy for reproduction, then running should extend maximum lifespan as does CR. However, running did not increase maximum lifespan (45, 46). Exercise did significantly increases the average lifespan of the rats, by about 10%. As we

discussed above, an increase in average lifespan can result from a reduction in disease incidence.

Since exercise increases the production of free radicals, damage in exercising animals might prevent exercise from extending maximum lifespan. However, exercise does not interfere with maximum lifespan extension in moderately CR rats (45, 46). Thus, exercise does not function like CR. Exercise increases average but not maximum lifespan. CR increases both average and maximum lifespan.

9. ARE THE EFFECTS OF CR DUE TO REDUCED LEVELS OF BODY FAT?

There is a direct relationship between adiposity (especially visceral fat mass) and mortality in humans. In 1960, Berg and Simms proposed that CR prolonged lifespan by reducing body fat (47). However, three subsequently published studies seem to clearly separate reduced body fat from the lifespan extending effects of CR. In the first study, rats which were calorie restricted during their first year of life and ad libitum fed thereafter were found to outlive rats that were ad libitum fed during the first year of life, and calorie restricted thereafter. The rats which were calorie restricted for only their first year of life had a greater fat mass for a longer period of time than the other group, yet they outlived the other group. From this, the authors concluded that depleted fat mass is not responsible for the effects of CR on lifespan.

In a second study, a longitudinal lifespan study, Bertrand et al. studied the relationship between body weight and lifespan. Even genetically identical rats will very widely in their weights if fed ad libitum. The authors found no relationship between body fat and length of life (48). In a third study, Harrison et al. found that genetically obese mice subjected to a CR diet had a greater maximum lifespan than ad libitum fed, genetically normal mice. This was true even though the CR obese mice had more than twice the body fat of the genetically normal ad libitum fed mice (49). Thus, though they were fatter, the CR obese mice outlived the thinner ad libitum mice.

In each of these studies, fat mass did not correlate with lifespan. Together, the studies indicate that reduced fat mass is not the source of the lifespan extending effects of CR, although there remains occasional debate on the matter (50, 51). Recently, Cleary et al. suggested that reduced fat mass is responsible for the extended lifespan of a mutant mouse strain termed "FIRKO mice" (52). FIRKO mice (fat insulin receptor knockout mice) do not express the insulin receptor in fat tissue. Other tissues have normal levels of the receptor. Despite having the same caloric intake on a per mouse basis as non-mutant, normal mice, FIRKO mice have less fat tissue and an 18% increase in median and maximum lifespans. The authors concluded that the extension of lifespan might be due to the reduction in fat mass. Their supposition may be correct. However, these data do not challenge the idea that CR extends lifespan by a mechanism other than reduced fat mass. We know that genetic mutations are capable of extending lifespan in mammals (see below). Thus, at present, the data suggest that depletion of fat mass is not responsible for the lifespan extension effects of CR.

10. WHAT IS THE MECHANISM OF ACTION OF CR?

There is no consensus regarding the mechanism of action of CR. Numerous theories have been put forward, and they are as diverse as the investigators who study them (Table 1). The oxidative stress hypothesis is perhaps the most popular of these theories. Harman originally proposed that the generation of reactive oxygen free radicals was the reason for aging (53). Mitochondria generate and release oxygen free radicals as a part of the process of converting food into the chemical energy used in metabolic processes. Other biological reactions such as fat oxidation and cytotoxic T-cell function also produce reactive oxygen species. Reactive oxygen species can oxidatively damage proteins, lipids, and nucleic acids. It is thought that this damage comes to outstrip repair processes as we age. In accord with this idea, oxidative damage accumulates with age. A number of investigators have proposed that CR may act by reducing oxidative stress in an organism, either by reducing the rate of oxidative damage or by increasing the rate of its repair (54, 55). CR does reduce the age-associated accumulation of oxidatively damaged lipids, proteins, and nucleic acids. But, until a causal link can be established between oxidative damage and aging, the validity of this hypothesis remains unproven. A number of the studies which seemed at first to strongly support the oxidative stress hypothesis were found to be due to experimental artifacts. In fact, no proposed mechanism for aging and the action of CR is presently well supported by direct evidence.

11. ARE THERE OTHER WAYS TO EXTEND LIFESPAN AND DELAY THE ONSET OF AGE-RELATED DISEASES?

Changes in the activity of specific genes can control the rate of aging and the rate of development of age-related diseases in invertebrates and

mammals (56, 57). In nematodes, lifespan is regulated by an insulin/insulin-like growth factor receptor homolog, DAY-2. Nematodes with mutations in this signal transduction pathway remain youthful longer, and live more than twice as long as non-mutants. In fruit flies (*Drosophila melanogaster*) a loss of function mutation in the insulin-like receptor homologue gene yields dwarf female flies with up to an 85% extension in adult longevity and dwarf male flies with reduced late age-specific mortality (58). These types of studies suggest that the insulin/insulin-like growth factor 1 pathway may have a significant role in aging.

This pathway also regulates the rate of aging in mammals (57). This is a remarkable example of an evolutionarily conserved molecular mechanism. According to the fossil and genetic records, nematodes and flies have not shared a common ancestor with mammals like mice and humans for about 570 million years. And yet, the insulin/insulin-like growth factor receptor signal transduction system remains important in determining lifespan in each species.

Recently, a small family of single gene mutations in mice which interfere with growth hormone/IGF-I signaling, resulting in dwarfism, have been shown to increase mean and maximal lifespans by 40% to 70% beyond those of their nonmutant siblings (57, 59, 60). These mice are homozygous for loss-of-function mutations in the Pitl (Snell dwarf mice), Propl (Ames dwarf mice), or GH receptor (GHR KO mice) loci. These mutants have in common reduced signaling through the insulin-like receptor signal transduction pathway. The mutations appear to slow the intrinsic rate of aging in mice. Snell dwarf mice show delays in age-dependent collagen cross linking and changes in six age-sensitive indices of immune system status (60). These findings demonstrate that a single gene can control maximum lifespan and the timing of senescence in mammals.

In another remarkable finding, the already enhanced lifespan of Ames dwarf mice was found to be further extended by -25% when the mutant mice were subjected to CR (61). Thus, at least two well-documented means of mammalian lifespan extension have been identified. And, the effects are additive. Together they are more effective. These observations suggest that we may not have yet observed the maximum enhancement possible in mammalian lifespan.

Dwarfism is an unexpected way to extend lifespan in mammals. We do not think of human dwarfs as especially healthy or long-lived. And this illustrates an important point. Both dwarf and CR mice are sensitive to the cold and have somewhat enhanced susceptibility to infectious disease. But, in the protected environment of a vivarium, each of these paradigms can extend lifespan. Of course, we are not mice in a vivarium. We have to deal with infectious disease, freeways, competition, the mortgage, and so on. We need to find a way to get the best effects of dwarfism and CR without the downsides. This is why some scientists, like myself, are working to develop and discover compounds that can mimic the beneficial effects of CR and the dwarf mutations.

12. WILL HUMANS RUSH TO EMBRACE THE PRACTICE OF CR?

We know from a plethora of published epidemiological and clinical studies that reduced calorie diets decrease the incidence of cardiovascular disease, type 2 diabetes, and even cancer in humans. And, there is the real possibility that CR may also be capable of extending the maximum lifespan of our species. So, you might expect that there would be a rush to embrace a CR lifestyle. In a sense there has been a rush to do so. Sales of weight reduction products of one type or another produce billions in profits for companies every year. However, almost none of these products are effective in the long run. The rate of recidivism after weight loss is nearly 100%. There are powerful genetic influences on our weight. Family studies demonstrate that obesity and thinness follow family lines (62). Twin and adoption studies indicate that weight variance in families is genetic in origin (63-65). Eating is so closely related to survival in evolution, that when we are hungry the message we receive from our unconscious mind is, "Eat now! Or you will die!" It does not matter what our weight is, the message is the same, "eat!". In fact, the obese probably receive this message more often, and with more urgency than the thin.

The problems with maintaining weight are well illustrated by the tragic story of Dr. Stuart M. Berger. He transformed himself from a lonely, fat child in Brooklyn into a trim, rich, celebrity nutrition doctor. He wrote the *Southampton Diet, Dr. Berger's Immune Power Diet,* and *Forever Young,* advocating among other things the preventative power of piles of steamed broccoli. Then he died in his sleep in his Manhattan apartment at the age of 40 and a weight of 365 pounds.

Dr. Berger knew what to do. He knew that obesity was unhealthy. But, in the end he could not keep that prompting from the unconscious to eat in check. To weigh less than our genes tell us to weigh, we probably must constantly fight that message to eat. This well illustrates why it is not very likely that we will soon be able to avail ourselves of the benefits of a CR diet through dieting. And, as discussed above, there is a downside to CR. Ideally we would like to avoid the reduced immune responsiveness and libido associated with CR.

So, as I mentioned above, a number of scientists are working to discover and develop compounds that will mimic the beneficial physiological effects of CR, without the diet. Such compounds have come to be termed, CR *mimetics*. During the past several years, my laboratory has been engaged in developing methods for the identification and development of CR mimetics.

13. ARE THERE PRESENTLY ANY AUTHENTIC ANTI-AGING PHARMACEUTICALS?

There are presently no authentic anti-aging pharmaceuticals. However, this by no means proves that they do not or cannot exist. The reason none have been found is that until recently no good assay has existed for identifying such mimetics. Appropriate assays are essential for identifying effective pharmaceuticals. For example, anti-cancer drugs were discovered before there was any understanding of neoplastic mechanisms. They were identified because drug screening protocols were developed using tumor models in cell culture and animals. No such assays have been available for screening anti-aging compounds until now.

Historically, the only accepted method of evaluating therapies that attempt to slow aging and the onset of age-related diseases has been lifespan studies. However, this method was also a formidable obstacle. Even a "short-lived" mammal like a mouse lives 40 months. Use of a shorter-lived, enfeebled rodent strain introduces confounds into the study. A cohort of at least 60 rodents is required to have the power to reliably detect a 10% change in longevity. Thus, largescale drug screening is impractical using this standard.

Gerontologists have recognized the need for a rapid assay for decades. For more than 25 years, they have been searching for biomarkers which would make it possible to detect the development of age-related diseases and the underlying rate of aging over short periods of time. The NIA spent 80 to 90 million dollars in the 1980s and early 1990s attempting to identify such biomarkers of aging (Dr. Huber Warner, NIA, personal communication). For the most part, these efforts have not met with much success.

Since changes in the activity of specific genes can control the rate of aging and slow the development of age-related diseases, a number of investigators have begun to apply microarray technology to the study of aging and CR (66-70). These studies allow us to measure the activity of thousands of genes simultaneously in any cell or tissue of interest. Commercial microarrays developed by Affymetrix Corporation have been used to measure the activity of 6- to 7-thousand genes in the gastrocnemius muscle, cerebral cortex, cerebellum, and hypothalamus of young and old mice, and old mice subjected to CR (68-70). In muscle, aging was accompanied by changes in gene expression linked to the development of the characteristic age-related pathologies of the tissue. These changes are associated with inflammation and stress, and with lower levels of expression of metabolic and biosynthetic genes. In the brain, aging appears to be accompanied by an increase in the inflammatory response and oxidative stress, and a reduction in neurotrophic support. CR appears to oppose many of these changes.

Our work over the past several years has convinced us that global gene expression profiles measured with high density microarrays provide the powerful assay required to rapidly develop and discover authentic CR mimetics.

14. MICROARRAYS PROVIDE AN ASSAY FOR DISCOVERING CR MIMETICS

Recently we published microarray expression analysis of approximately 12,000 genes in the liver of young and old mice subjected to long-term CR (LT-CR) and ST-CR (67). ST-CR appeared to rapidly reverse approximately 70% of the age-related gene expression changes which were also reversed by LT-CR (Fig. 4). The changes induced by ST-CR shifted the gene expression profile of very old mice closer to that of young mice. In addition, both LT-CR and ST-CR also produced changes in the expression of a sizable group of other genes which did not change with age. These changes were in categories of genes which were very similar to the age-responsive genes. Altogether, LT- and ST-CR produced changes in gene expression consistent with increased protein turnover, healthful changes in apolipoprotein and fatty acid biosynthesis, enhanced anti-proliferative growth control (an anti-cancer effect), increased apoptosis (another anti-cancer effect) and reduced chemical carcinogenesis (again, this effect is anti-cancer).

Our studies suggest that rather than simply preventing age-related changes in gene expression, CR instead acts rapidly to establish a new profile of gene expression which may better resist aging. Importantly, ST-CR produced a profile of gene expression effects which substantially reproduced the effects of LT-CR, but in only 4 weeks.

15. ARE THE PHYSIOLOGICAL EFFECTS OF CR ALSO RAPIDLY INDUCIBLE?

As discussed above, the effectiveness of CR appears to increase proportionally with its duration (5). Beauchene et al. used intermittent feeding to produce CR in rats (42). They found that one year of CR was effective in extending lifespan whether it was applied only during the first year of life, and the animals were ad libitum fed thereafter. CR was also effective if the animals were CR fed after a first year of ad libitum feeding. Life-long CR was more effective than one year of CR, regardless of when it was applied. These results suggest that CR extends lifespan only while it is applied, whether it is continuous or applied only during a part of the lifespan of an animal.

Consistent with this idea, Higami et al. found that the duration of CR, and not whether CR was intermittent or continuous, correlated closely with the incidence and age of onset of leukemia in F344 rats (71). These results suggest that the anti-disease effects of CR are cumulative, and accrue anytime the CR diet is applied.

Sohal and his colleagues performed dietary crossover studies in aged calorie restricted and ad libitum fed mice (72, 73). They found an age-related increase in protein oxidative damage measured as increased carbonyl concentration and decreased sulfhydryl content in homogenates of brain and heart. These changes were reduced in CR animals. Importantly, carbonyl content of the whole brain and the sulfhydryl content of the heart were reversible by 3 to 6 weeks of STCR or short-term control feeding. These findings suggest that the beneficial effects of LT-CR on brain function also might be rapidly reversible. That is, ST-CR might rapidly reduce the steadystate levels of oxidative stress, improving brain function. Likewise, a shift from LT-CR to the control diet might rapidly dissipate the neuroprotective effects of a life time of CR on brain function. The weakness in interpreting these results is that oxidative damage to proteins in the brain has not been shown to influence cognition.

Goto et al. found that two months of CR in old mice significantly reduced the heat liability of proteins in the liver, kidney, and brain, and reversed the age-associated increase in the half-life of proteins (74). ST-CR also reduced carbonylated proteins in liver mitochondria in old rats. These results suggest that CR rapidly reduces the dwell time of the proteins by promoting protein turnover. Consistent with this idea, proteasome activity increased rapidly in the liver of old ST-CR rats.

In another study, some oxidative damage in old animals was not rapidly reversed by LTCR. Sohal and his colleagues found that mitochondria in skeletal muscles accumulate significant amounts of oxidative damage during aging, and this damage does not accumulate in LT-CR mice (75). Results from crossover studies indicated that mitochondrial oxidative damage in muscle could not be reversed by 6 weeks of CR. However, other time points were not studied. Therefore, the kinetics of the potential physiological shift between dietary states remain unknown in muscle.

16. HOW CAN WE IDENTIFY AND DEVELOP AUTHENTIC CR MIMETICS?

Together, these studies and our microarray results indicate that CR acts rapidly to produce a physiological state associated with health and longevity in mammals, including humans.

These observations have important implications for the search for authentic anti-aging treatments. They indicate that relatively brief courses of treatment with a candidate CR mimetic should be capable of rapidly and substantially reproducing the gene expression, lifespan extending and prophylactic effects of CR. We are finding success using this approach to screen for CR mimetics.

17. CONCLUSION

CR is a well-established nutritional paradigm that extends average and maximum lifespans and reduces the incidence and age of onset of most age-related diseases. CR does this in a phylogenetically diverse group of organisms, including mammals. The effects of CR on physiology and lifespan result from reduced calorie intake. The effects are independent of diet composition, provided that malnutrition is avoided. There is suggestive evidence that CR reduces age-related disease and extends lifespan in non-human primates, and perhaps in humans. These remarkable effects apparently arose to respond to boom and bust nutritional cycles. It allows scarce nutritional resources to be directed away from reproduction, and into physiological maintenance and survival during times of nutritional stress. The mechanism used by CR to exert its remarkable effects remains a mystery. Aerobic exercise can extend average lifespan, but not maximum lifespan, suggesting that it does not mimic the effects of CR. The reduction in body fat in CR animals does not appear to be responsible for the life-extension effects. However, the additive effects of CR and the dwarf mutations on the lifespan of mice suggest that the limits of lifespan extension possible in mammals have not been found. Because not many humans are able to consistently under-eat, there is a need for pharmaceuticals that can mimic the effects of CR. Genomic profiling of LT- and ST-CR using microarrays has revealed gene expression biomarkers

which can be used to rapidly discover and develop drugs capable of reproducing the health and longevity benefits of CR.

18. REFERENCES

1. Olshansky SJ, Carnes BA, Desesquelles A, Demography. Prospects for human longevity, Science, 291, 1491-1492, 2001.
2. McCay CM, Crowell MF, Maynard LA, The effect of retarded growth upon the length of the life span and upon the ultimate body size, J Nutr, 10, 63-79, 1935.
3. Ross MH, Length of life and nutrition in the rat, J Nutr, 75, 197-210, 1961.
4. Weindruch R, Walford RL, The Retardation of Aging and Disease by Dietary Restriction, Springfield, IL, Charles C. Thomas, 1988.
5. Merry BJ, Molecular mechanisms linking calorie restriction and longevity, Int J Biochem Cell Biol, 34, 1340-1354, 2002.
6. Weindruch R, Walford RL, Fligiel S, Guthrie D, The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake, J Nutr, 116, 641-654, 1986.
7. Masoro EJ, Assessment of nutritional components in prolongation of life and health by diet, Proc Soc Exp Biol Med, 193, 31-34, 1990.
8. Roth GS, Ingram DK, Black A, Lane MA, Effects of reduced energy intake on the biology of aging: the primate model, Eur J Clin Nutr, 54 Suppl 3, S 15-S20, 2000.
9. Dhahbi JM et al, Caloric restriction alters the feeding response of key metabolic enzyme genes, Mech Ageing Dev, 122, 35-50, 2001.
10. Lane MA et al, Diet restriction in rhesus monkeys lowers fasting and glucose-stimulated glucoregulatory end points, Am J Physiol, 268, E941-E948, 1995.
11. Bodkin NL, Ortmeyer HK, Hansen BC, Long-term dietary restriction in older-aged rhesus monkeys: effects on insulin resistance, J Gerontol A Biol Sci Med Sci, 50, B 142-B 147, 1995.
12. Kemnitz JW, Roecker EB, Weindruch R, Elson DF, Baum ST, Bergman RN, Dietary restriction increases insulin sensitivity and lowers blood glucose in rhesus monkeys, Am J Physiol, 266, E540-E547, 1994.
13. Lane MA, Ingram DK, Roth GS, Calorie restriction in nonhuman primates: effects on diabetes and cardiovascular disease risk, Toxicol Sci, 52, 41-48, 1999.
14. Kealy RD et al, Effects of diet restriction on life span and age-related changes in dogs, J Am Vet Med Assoc, 220, 1315-1320, 2002.
15. Rudzinska MA, Overfeeding and lifespan in Tokophyra Infusionum, J Gerontol, 7, 544, 1952.
16. Austad SN, Life extension by dietary restriction in the bowl and doily spider, Frontinella pyramitela, Exp Gerontol, 24, 83-92, 1989.
17. Hayflick L, The future of ageing, Nature, 408, 267-269, 2000.
18. Roth GS, Ingram DK, Lane MA, Calorie restriction in primates: will it work and how will we know?, J Am Geriatr Soc, 47, 896-903, 1999.
19. Lane MA, Ingram DK, Cutler RG, Knapka JJ, Barnard DE, Roth GS, Dietary restriction in nonhuman primates: Progress report on the NIA study, Ann NY Acad Sci, 673, 36-45, 1992.
20. Kemnitz JW, Weindruch R, Roecker EB, Crawford K, Kaufman PL, Ershler WB, Dietary restriction of adult male rhesus monkeys: design, methodology, and preliminary findings from the first year of study, J Gerontol, 48, B 17-1326, 1993.
21. Hansen BC, Bodkin NL, Primary prevention of diabetes mellitus by prevention of obesity in monkeys, Diabetes, 42, 1809-1814, 1993.
22. Hansen BC. Efficacy of Long Term Calorie Restriction of Adult Monkeys to Lower Risk of Death and Reduced Morbidity. FASEB.Orlando, FL, 2001, 400 . 2002. Ref Type: Abstract
23. Verdery RB, Ingram DK, Roth GS, Lane MA, Caloric restriction increases HDL2 levels in rhesus monkeys (Macaca mulatta), Am J Physiol, 273, E714-E719, 1997.
24. Cefalu WT et al, In vitro oxidation of low-density lipoprotein in two species of nonhuman primates subjected to caloric restriction, J Gerontol A Biol Sci Med Sci, 55, 13355-13361, 2000.
25. Lane MA et al, Short-term calorie restriction improves disease-related markers in older male rhesus monkeys (Macaca mulatta), Mech Ageing Dev, 112, 185-196, 2000.
26. Weyer C et al, Energy metabolism after 2 y of energy restriction: the biosphere 2 experiment, Am J Clin Nutr, 72, 946-953, 2000.
27. Walford RL, Mock D, MacCallum T, Laseter JL, Physiologic changes in humans subjected to severe, selective calorie restriction for two years in biosphere 2: health, aging, and toxicological perspectives, Toxicol Sci, 52, 61-65, 1999.
28. Verdery RB, Walford RL, Changes in plasma lipids and lipoproteins in humans during a 2year period of dietary restriction in Biosphere 2, Arch Intern Med, 158, 900-906, 1998.
29. Roth GS et al, Biomarkers of caloric restriction may predict longevity in humans, Science, 297, 811, 2002.
30. Eveleth PB, Tanner JM, World Wide Variation in Human Growth, New York, Cambridge University Press, 1990.
31. Ellison PT, Age and developmental effects on adult ovarian function, In Rosetta L, Mascie-Taylor NCG, eds, Variability in Human Fertility: A Biological Anthropological Approach, New York, Cambridge University Press, pp. 69-90, 1996.
32. Prentice AM, Lunn PG, Watkinson M, Whitehead RG, Dietary supplementation of lactating Gambian women. II. Effect on maternal health, nutritional status and biochemistry, Hum Nutr Clin Nutr, 37, 65-74, 1983.
33. Lager C, Ellison PT, Effects of moderate weight loss on ovarian function assessed by salivary progesterone measurements, Am J Human Biol, 2, 303-312, 1990.
34. Lee RB, The !Kung San: Men, Women, and Work in a Foraging Society, Chicago, IL, University of Chicago Press, 1979.
35. Somes GW, Kritchevsky SB, Shorr RI, Pahor M, Applegate WB, Body mass index, weight change, and death in older adults: the systolic hypertension in the elderly program, Am J Epidemiol, 156, 132-138, 2002.
36. Pasanisi F, Contaldo F, de Simone G, Mancini M, Benefits of sustained moderate weight loss in obesity, Nutr Metab Cardiovasc Dis, 11, 401-406, 2001.
37. Kagawa Y, Impact of Westernization on the nutrition of Japanese: changes in physique, cancer, longevity and centenarians, Prev Med, 7, 205-217, 1978.
38. Keys A, Brozek J, Henschel A, Mickelson O, Taylor H, The Biology of Human Starvation, Minneapolis, MN, University of Minnesota Press, 1950.
39. Stunkard AJ, Nutrition, aging and obesity, In Rockstein M, Sussman ML, eds, Nutrition, longevity, and aging, New York, Academic Press, pp. 253-284, 1976.
40. Walford RL, Mock D, Verdery R, MacCallum T, Calorie Restriction in Biosphere 2: Alterations in Physiologic, Hematologic, Hormonal, and Biochemical Parameters in Humans Restricted for a 2-Year Period, J Gerontol A Biol Sci Med Sci, 57, B211-B224, 2002.
41. Masoro EJ, Austad SN, The evolution of the antiaging action of dietary restriction: A hypothesis, J Gerontol : Biol Sci, 51A, B387-B391, 1996.
42. Beauchene RE, Bales CW, Bragg CS, Hawkins ST, Mason RL, Effect of age of initiation of feed restriction on growth, body composition, and longevity of rats, J Gerontol, 41, 1319, 1986.

43. Miller RA, Harper JM, Dysko RC, Durkee SJ, Austad SN, Longer life spans and delayed maturation in wild-derived mice, Exp Biol Med (Maywood), 227, 500-508, 2002.
44. Holloszy JO, Smith EK, Vining M, Adams S, Effect of voluntary exercise on longevity of rats, J Appl Physiol, 59, 826-831, 1985.
45. Holloszy JO, Schechtman KB, Interaction between exercise and food restriction: effects on longevity of male rats, J Appl Physiol, 70, 1529-1535, 1991.
46. Holloszy JO, Mortality rate and longevity of food-restricted exercising male rats: a reevaluation, J Appl Physiol, 82, 399-403, 1997.
47. Berg BN, Simms HS, Nutritional and longevity in the rat. II. Longevity and onset of disease with different levels of food intake., J Nutr, 71, 255-263, 1960.
48. Bertrand HA, Lynd FT, Masoro EJ, Yu BP, Changes in adipose mass and cellularity through the adult life of rats fed ad libitum or a life-prolonging restricted diet, J Gerontol, 35, 827-835, 1980.
49. Harrison DE, Archer JR, Astle CM, Effects of food restriction on aging: Separation of food intake and adiposity., Proc Natl Acad Sci U S A, 81, 1835-1838, 1984.
50. Barzilai N, Gupta G, Revisiting the role of eat mass in the life extension induced by caloric restriction, J Gerontol : Biol Sci, 54, B89-B96, 1999.
51. Masoro EJ, Commentary on "Revisiting the Role of Fat Mass in the Life Extension Induced by Caloric Restriction", Journal of Gerontology: Biological Sciences, 54A, B97, 1999.
52. Bluher M, Kahn BB, Kahn CR, Extended longevity in mice lacking the insulin receptor in adipose tissue, Science, 299, 572-574, 2003.
53. Harman D, Aging: a theory based on free radical and radiation chemistry, J Gerontol, 11, 298-300, 1956.
54. Sohal RS, Weindruch R, Oxidative stress, caloric restriction, and aging, Science, 273, 5963, 1996.
55. Yu BP, Aging and oxidative stress: modulation by dietary restriction, Free Radic Biol Med, 21, 651-668, 1996.
56. Guarente L, Kenyon C, Genetic pathways that regulate ageing in model organisms [In Process Citation], Nature, 408, 255-262, 2000.
57. Brown-Borg HM, Borg KE, Meliska CJ, Bartke A, Dwarf mice and the ageing process [letter], Nature, 384, 33, 1996.
58. Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS, A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function, Science, 292, 107-110, 2001.
59. Coschigano KT, Clemmons D, Bellush LL, Kopchick JJ, Assessment of growth parameters and life span of GHR/BP gene-disrupted mice, Endocrinology, 141, 2608-2613, 2000.
60. Flurkey K, Papaconstantinou J, Miller RA, Harrison DE, Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production, Proc Natl Acad Sci U S A, 98, 6736-6741, 2001.
61. Bartke A, Wright JC, Mattison JA, Ingram DK, Miller RA, Roth GS, Extending the lifespan of long-lived mice, Nature, 414, 412, 2001.
62. Maes HH, Neale MC, Eaves LJ, Genetic and environmental factors in relative body weight and human adiposity, Behav Genet, 27, 325-351, 1997.
63. Stunkard AJ et al, An adoption study of human obesity, N Engl J Med, 314, 193-198, 1986.
64. Stunkard AJ, Harris JR, Pedersen NL, McClearn GE, The body-mass index of twins who have been reared apart, N Engl J Med, 322, 1483-1487, 1990.
65. Sorensen TI, Price RA, Stunkard AJ, Schulsinger F, Genetics of obesity in adult adoptees and their biological siblings, BMJ, 298, 87-90, 1989.
66. Han E, Hilsenbeck SG, Richardson A, Nelson JF, cDNA expression arrays reveal incomplete reversal of age-related changes in gene expression by calorie restriction, Mech Ageing Dev, 115, 157-174, 2000.
67. Cao SX, Dhahbi JM, Mote PL, Spindler SR, Genomic profiling of short- and long-term caloric restriction in the liver of aging mice, Proc Natl Acad Sci U S A, 98, 10630-10635, 2001.
68. Jiang CH, Tsien JZ, Schultz PG, Hu Y, The effects of aging on gene expression in the hypothalamus and cortex of mice, Proc Natl Acad Sci U S A, 98, 1930-1934, 2001.
69. Lee CK, Weindruch R, Prolla TA, Gene-expression profile of the ageing brain in mice, Nat Genet, 25, 294-297, 2000.
70. Lee CK, Klopp RG, Weindruch R, Prolla TA, Gene expression profile of aging and its retardation by caloric restriction, Science, 285, 1390-1393, 1999.
71. Higami Y, Yu BP, Shimokawa I, Masoro EJ, Ikeda T, Duration of dietary restriction: an important determinant for the incidence and age of onset of leukemia in male F344 rats, J Gerontol, 49, B239-B244, 1994.
72. Dubey A, Forster MJ, Lal H, Sohal RS, Effect of age and caloric intake on protein oxidation in different brain regions and on behavioral functions of the mouse, Arch Biochem Biophys, 333, 189-197, 1996.
73. Forster MJ, Sohal BH, Sohal RS, Reversible effects of long-term caloric restriction on protein oxidative damage, J Gerontol A Biol Sci Med Sci, 55, B522-B529, 2000.
74. Goto S, Takahashi R, Araki S, Nakamoto H, Dietary Restriction Initiated in Late Adulthood Can Reverse Age-related Alterations of Protein and Protein Metabolism, Ann N Y Acad Sci, 959, 50-56, 2002.
75. Lass A, Sohal BH, Weindruch R, Forster MJ, Sohal RS, Caloric restriction prevents ageassociated accrual of oxidative damage to mouse skeletal muscle mitochondria, Free Radic Biol Med, 25, 1089-1097, 1998.

TABLE 1. PROPOSED MECHANISMS OF ACTION OF CR

1. Prevents Deleterious Age-related Changes in Gene Expression?

2. Reduces Oxidative Stress and Oxidative Damage?

3. Changes in Energy Metabolism?

4. Reduces Damage caused by Glucose (Glycation and Glycoxidation)?

5. Reduces Body Temperature?

6. Reduces DNA Damage or Increases DNA Repair?

7. Causes Neuroendocrine Changes which Extend Lifespan?

8. Slows the Rate of Telomere Shortening?