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Dietary Restriction: An Experimental Approach to the Study of the Biology of Aging

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I. Introduction

It is more than 60 years since McCay and his colleagues (McCay *et al.*, 1935) published their landmark paper showing that restricting food intake of rats soon after weaning increases the length of life. In the ensuing years, this finding has been confirmed repeatedly. In addition, the life-extending action has also been shown in mice and hamsters as well as in nonmammalian species, such as fish, flies, and water fleas. The life extension appears to be due to the slowing of the aging processes. Indeed, this phenomenon, which is often referred to as the antiaging action of dietary restriction (DR) or caloric restriction (CR), has been and is one of the most active areas of research in biological gerontology. Detailed coverage of the research on DR prior to 1993, including references to the original literature, can be found in a review article by Masoro (1993) and an encyclopedic book by Weindruch and Walford (1988). This chapter will focus on more recent findings; however, earlier work will be discussed as needed to provide a proper context.

II. Rodent Models

Studies on rats and mice, the animal models most used in DR research, have provided detailed information on DR's antiaging actions, as well as insights on possible underlying biological mechanisms. In these studies, the food intake of the DR group ranged from 50–70% of that of a control group; in most but not all studies, the control groups were fed *ad libitum*.

A. Antiaging Actions of Dietary Restriction

The mortality characteristics of populations of rats and mice undergoing DR have provided strong support for an antiaging action. The effects of DR on age changes in physiological processes and on the occurrence and progression of age-associated diseases have supplied additional support.

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1. *Mortality Characteristics*

As already stated, the findings of McCay *et al.* (1935) have been confirmed repeatedly in these numerous studies, both genders of several different genotypes of rats and mice have been used. In this regard, a study on the influence of DR on the survival characteristics of three rat genotypes and four mouse genotypes simultaneously maintained at the National Center for Toxicological Research in Jefferson, AR, is particularly impressive (Turturro *et al.*, 1999).

In addition, it has been found that life prolongation is robust even when DR is initiated in young adult life rather than soon after weaning (Yu *et al.*, 1985). Indeed, it has been found that DR initiated during early middle age significantly increases longevity in mice, though not as markedly as when started at earlier ages (Weindruch & Walford, 1982). This finding makes it clear that life prolongation by DR is not secondary to the prolongation of immaturity, a view initially proposed by McCay and his colleagues and long held by many. However, it is important to note that findings show that DR is not effective in prolonging life when initiated during late middle age or old age; when DR was started at 18 or 26 months of age in F344 × BN F1 hybrid male rats, there was no increase in longevity (Lipman *et al.*, 1998).

In addition to reducing the amount of food eaten, DR also changes the temporal pattern of food intake, with a meal-eating pattern replacing a nibbling pattern. This raises the possibility that the altered pattern of food intake rather than the reduced amount of food consumption is responsible for the antiaging action. This possibility was investigated, and the findings clearly show that reduced food intake is the factor responsible (Masoro *et al.*, 1995).

Gompertzian analyses of mortality characteristics of *ad libitum* fed and dietary restricted rat populations also strongly support the view that life prolongation by DR is due to its antiaging action. The analysis of mortality data from four rat studies, in which ingested food was about 60% of the *ad libitum* intake, showed that the mortality rate doubling time of the *ad libitum* fed rats ranged from 99–104 days and that of the dietary restricted rats ranged from 187–210 days (Holehan & Merry, 1986).

2. *Physiological Functions*

At advanced ages, most physiological processes of mice and rats on a DR regimen remain in a youthful state. Indeed, the number of functions thus affected by DR is so great that it is neither possible nor appropriate to provide anything approaching an encyclopedic coverage in a chapter of this length. Rather, our discussion will be limited to those effects that, in the author's opinion, may play an important part in the antiaging action.

DR modulates several fundamental cellular processes that may be intimately involved in aging. For example, it is quite likely that damage to DNA plays an important role in aging. In response to ultraviolet irradiation damage, DNA repair decreases with age in mouse splenocytes (Licastro *et al.*, 1988) and in rat liver and kidney cells (Weraarchakul *et al.*, 1989). It is significant that, in these studies, DR was found to retard the age-related decline in DNA repair activity. There is suggestive evidence that DR has a similar action in mouse skin cells (Lipman *et al.*, 1989). Also, cultured hepatocytes from old rats exhibit a compromised coupling of transcription and DNA repair, an age change that is prevented by DR (Guo *et al.*, 1998a,b). It appears that the effect of DR on DNA repair depends on the type of DNA damage (Haley-Zitlin & Richardson, 1993). Indeed, a study

indicates that the ability of DR to increase DNA repair is not universal; activities of DNA polymerases (enzymes involved in DNA repair) were found to be increased by DR in some, but not all, brain regions of the rat (Prapurna & Rao, 1996).

There is other evidence that DR helps maintain the stability of the nuclear and mitochondrial genomes with increasing age. DR markedly decreases the age-associated accumulation of mutations at the hypoxanthine phosphoribosyl transferase locus in mice (Dempsey *et al.*, 1993). Also, DR initiated in rats at middle age decreases the accumulation of skeletal muscle mitochondrial deletions and enzyme abnormalities and retards the loss of muscle fibers (Aspnes *et al.*, 1997).

Apoptosis eliminates damaged cells from the organism, and by so doing may protect the organism from deteriorative aspects of aging. Thus, it is significant that DR has been found to promote apoptosis in the liver of aging mice (Muskhelishvilli *et al.*, 1995) and the small intestine and colon of aging rats (Holt *et al.*, 1998). Moreover, apoptosis of preneoplastic cells is enhanced preferentially by DR in rats, thereby protecting them from carcinogenesis (Grasl-Kraup *et al.*, 1994). James and Muskhelishvili (1994) have also linked DR's increase in hepatic apoptosis in mice to a decreased incidence in hepatoma. On the other hand, DR prevents the age-associated increase in the susceptibility of rat hepatocytes to cell death induced by the administration of cycloheximide (Higami *et al.*, 1996).

Damaged proteins accumulate in cells with increasing age, which may well negatively impact cellular function. By degrading such proteins, proteolytic enzymes act to lessen this accumulation. However, protein degradation decreases with increasing age (Van Remmen *et al.*, 1995). DR attenuates the age-associated decrease in proteolysis (Ward, 1988), and this action may well be an important component of its antiaging action. It was suggested that DR's modulation of the age change in proteolysis might be due to alterations in proteosomes; however, studies designed to address this issue indicate that such is not the case (Shibatani *et al.*, 1996; Scrofano *et al.*, 1998).

The functioning of cells is regulated by hormones, cytokines, and neurotransmitters, which bind to cell receptors and alter cellular function by a complex pathway of interlinked chemical reactions collectively referred to as cellular signal transduction. Change with age in receptors and/or signal transduction could cause cellular dysfunction, and such changes do occur with increasing age. For example, cholinergic and dopaminergic stimulation of the formation of inositol phosphates (signal transduction pathway components) is decreased with increasing age in the brain of male F344 rats, and this decrease is prevented by DR (Undie & Friedman, 1993). Also, DR prevents the age-associated impairment in rat brain of the mitogen-activated protein kinase (MAPK) signal pathways (Zhen *et al.*, 1999).

With increasing age, there is a progressive impairment of the signal transduction pathway for growth hormone; DR was found to delay this impairment in mice (Xu & Sonntag, 1996a). Specifically, growth hormone activation of Stat–3 decreases with age, and DR prevents this decrease (Xu & Sonntag, 1996b). Is this action of DR important in its antiaging action? DR retards the age-associated decrease in protein biosynthesis (Van Remmen *et al.*, 1995). DR has been shown to increase the amplitude of growth hormone pulses in old animals (Sonntag *et al.*, 1995) and, by so doing, to sustain insulin-like growth factor-1 (IGF-1), which, in turn, sustains protein synthesis. By delaying age-associated impairment of the signal

transduction pathway for growth hormone, DR probably enables increased amplitude of growth hormone pulses to effectively maintain IGF-1 production. This action and the increase by DR of type 1 IGF receptors are probably the major factors involved in the promotion of protein synthesis by DR (D'Costa *et al.*, 1993).

A key process in many cellular signal transduction pathways is the mobilization of intracellular calcium stores, thus increasing the concentration of cytoplasmic calcium ion. The ability of epinephrine to increase cytoplasmic calcium ion concentration in parotid acinar cells declines with advancing age in male F344 rats; DR blunts, but does not prevent, this decline (Salih *et al.*, 1997).

The limited amount of research indicates that modulating age changes in cellular signal transduction may be an important aspect of the antiaging action of DR. Further research in this area should be given high priority.

Because changes in gene expression occur with advancing age, at least some of these changes are likely to play an important role in aging. On the basis of the levels of mRNA transcripts as an index, the expression of some genes decreases and that of others increases, whereas the expression of many other genes does not change with increasing age. For example, the level of mRNA transcripts for both catalase and superoxide dismutase in the liver decrease with age, and DR counters this change (Van Remmen *et al.*, 1995).

The induction of hsp70 in rat hepatocytes in response to heat is the result of increased transcription. This induction, which decreases with increasing age, is enhanced at all ages by DR, an enhancement that correlates with an increase in binding of its transcription factor (Heydari *et al.*, 1993). However, DR decreases the expression of Er_p57, Er_p72, GRP170, calreticulin, and calnexin in mouse liver (Dhahbi, *et al.*, 1997). DR was found to blunt the age-associated decrease in IL–2 expression in rat splenic T-cells and to increase the binding activity of transcription factor NFAT and the expression of c-fos, a component of the NFAT–protein complex (Pahlavani *et al.*, 1997).

The induction in the fasting state of hepatic gluconeogenic phosphoenolpyruvate carboxykinase decreases with advancing age, and DR prevents this decrease (Van Remmen & Ward, 1998). Whereas the synthesis of p^{53} and its phosphorylation in response to retinoic acid, which damages DNA, decreases with age, DR prevents this decrease (Pipkin *et al.*, 1997).

The information on the relation of gene expression, aging, and DR still is quite limited. Nevertheless, it is already clear that the influence of DR on gene expression increases the ability of the aging rodent to cope more effectively with challenges.

The neuroendocrine system (i.e., the hypothalamic–pituitary system) is a key player in the regulation of organismic function, and thus age-associated alterations in neuroendocrine function could have a major role in aging. Indeed, the neuroendocrine system is known to be involved in the aging of the female rodent's reproductive system. DR delays age-related loss of estrous cycles and promotes gonadotropin secretion by the adenohypophysis of aging female rats (McShane & Wise, 1996). In the female rat arcuate nucleus, DR differentially influences the expression of neuropeptides that regulate reproductive function, increasing neuropeptide Y mRNA per cell, decreasing proopiomelanocortin mRNA per cell, and not changing galanin mRNA per cell (McShane *et al.*, 1999).

DR also influences the neuroendocrine function in male rats. Within weeks of its initiation in young male rats, DR was

found to decrease the mRNA content in the pituitary of luteinizing hormone, follicle-stimulating hormone, thyrotrophin, growth hormone, and prolactin, but not that of proopiomelanocortin (Han *et al.*, 1998). Whereas it is obvious that such alterations in pituitary function are almost certain to influence the functioning of many different cell types throughout the body, it is important that this work be extended to include old animals in which DR has been initiated at an early age. Although DR decreases growth hormone pulse amplitude in young rats, long-term DR increases the amplitude in old rats (Sonntag *et al.*, 1995); long-term DR acts by decreasing the translation of hypothalamic somatostatin–mRNA and, thus, the secretion of somatostatin into the hypothalamic–adenohypophyseal portal blood and possibly also by increasing the secretion of hypothalamic growth-hormone-releasing hormone. Long-term DR also increases the number of functional pituitary somatotropes (Shimokawa *et al.*, 1996a). Indeed, Sonntag *et al.*, (1999) propose that, by increasing growth hormone at advanced ages and maintaining low levels of IGF-I thoughout life, DR decreases pathologies secondary to dysregulation of cell replication and thereby increases life span.

Much more research is needed on the relation of the neuroendocrine system to aging and its modulation by DR. What has been done to date indicates that such studies will be rewarding, particularly if they are carried out in conjunction with studies on cellular signal transduction pathways, because the action of the hormones of the neuroendocrine system on target cells is dependent on the functioning of these pathways.

Aging of the cardiovascular system causes functional problems for many elderly people. An age-associated loss in the response of the heart to β-adrenergic stimulation is one of these functional problems. This decrease in responsiveness also occurs in the hearts of aging rats, an effect that is countered by DR (Kelley & Herhihy, 1998). Also, studies using membrane preparations from rat ventricular muscle show an age-associated decline in isoproterenol-stimulated adenylyl cyclase activity, and this is attenuated by DR (Gao *et al.*, 1998). In addition, aging is known to reduce the capacity of cardiac adrenergic nerve terminals of male rats to release norepinephrine, and this reduced capacity is attentuated by DR (Snyder *et al.*, 1998).

Deterioration of the immune system occurs in humans and animal models with increasing age, and many gerontologists believe that this plays an important role in organismic aging. DR retards much, but not all, of this deterioration in mice and rats (Miller, 1995). For example, DR retards the age-related decline in the immune response of mice to the influenza A virus (Effros *et al.*, 1991); specifically, the decline in antigen presentation, T-cell proliferation, and antibody production in old mice is attenuated by a long-term DR regimen. Studies provide some insight on mechanisms underlying this protection. DR enhances the apoptotic elimination of nonfunctional T-cells in old mice (Spaulding *et al.*, 1997). In addition, DR delays the decline with age in circulating naive T-cells and in immature T-cell precursors in the thymus (Chen *et al.*, 1998). The extensive literature on the effect of DR on the immune system is available in a review by Pahlavani (1998).

Wound healing in rodents is impaired with increasing age, and long-term DR does not to retard this impairment (Reiser *et al.*, 1995; Roth *et al.*, 1997). A study on mice has shown that DR actually does maintain the youthful capacity to heal wounds, but for this action to be manifest requires an abundant intake of dietary energy just prior to and following the

wounding (Reed *et al.*, 1996). Specifically, when provided with *ad libitum* feeding starting 4 weeks prior to wounding, old DR mice heal as rapidly as young *ad libitum* fed mice and much more rapidly than old mice fed *ad libitum* for their entire life. This increased capacity for wound repair appears to relate to an enhancement of collagen biosynthesis and cell proliferation. In regard to the latter, six cell types of male B6D2F1 mice have shown a diminished ability to proliferate by middle age. Whereas DR prevents this decrease in proliferative ability, 4 weeks of *ad libitum* feeding are required for this effect to be manifest (Wolf *et al.*, 1995).

3. *Age-associated Diseases*

From the late 1930s to the present time, many studies have shown that DR delays or, in some instances, prevents the occurrence of most age-associated diseases. These studies have involved many different rat and mouse genotypes, some with unique age-associated disease processes. The spectrum of such disease processes retarded by DR includes many different spontaneous neoplasms, degenerative diseases such as nephropathy and cardiomyopathy, and autoimmune diseases. For articles that summarize these findings, including citations of the original work, see Keenan *et al.*, (1995a,b), Lipman *et al.*, (1999a), Masoro (1993), and Weindruch and Walford (1988). A paper comparing the influence of DR on age-related lesions in three rat genotypes (F344, BN, and BNF3F1) is particularly informative (Lipman *et al.*, 1999b). Thus, there is no need to review this information in the present chapter, other than to point out a mechanistic study on the anticarcinogenic action of DR. On the basis of a study of bladder cancer in mice, Dunn *et al.*, (1997) propose that DR slows the progression of cancers by decreasing IGF-1 levels, thereby favoring apoptosis over cell proliferation.

The following studies are also worth mentioning. DR slows the development of lens cataracts in the cataract-prone Emory mouse strain (Taylor *et al.*, 1995). DR also retards the age-associated decrease in the proliferative capacity of mouse lens epithelial cells (Li *et al.*, 1997); whether this action plays a role in slowing cataract formation remains to be determined. In addition, DR was found to decrease blood pressure in spontaneously hypertensive, stroke-prone (SHR-SP) rats (Stevens *et al.*, 1998).

B. Nutritional Factor Responsible for Antiaging Action

The antiaging action of DR could be due to decreased intake of a specific nutrient or dietary contaminant or to reduced energy intake. Although manipulation of specific nutrients, such as reducing the intake of protein (Yu *et al.*, 1985), replacing casein with soy protein (Iwasaki *et al.*, 1988), or replacing sucrose with cornstarch (Murtagh-Mark *et al.*, 1995), can influence life span, their effects are small compared to those of DR. The use of semisynthetic diets to evaluate the effect of decreasing specific components has also provided strong evidence that it is the reduction in energy intake that is the major dietary factor underlying the life-prolonging and anticarcinogenic actions of DR (Masoro, 1992; Shimokawa *et al.*, 1996b). Indeed, it is for this reason that dietary restriction often is referred to as caloric restriction (CR).

It is unlikely that the reduced intake of a dietary toxic contaminant is significantly involved in the antiaging action. The findings with the semisynthetic diets, in which the amount of specific dietary components is manipulated as well as the many different food sources used in the different studies, just about preclude such a possibility.

C. Proposed Mechanisms of Antiaging Action

In their initial report, McCay *et al.* (1935) proposed a mechanism for the life-prolonging action of DR. Since then, many other mechanisms have been proposed. This keen interest in mechanism relates to the insights this knowledge could yield on the basic nature of aging and to the potential database it should provide for the development of antiaging interventions in humans.

1. *Overview of Proposed Mechanisms*

McCay *et al.* (1935) proposed that DR increased longevity by retarding growth and development. This view was held widely for quite some time, but further study found DR to be effective in extending life even when not initiated until young adulthood. Since then, other mechanisms have been proposed, many of which focus on the effect of DR on a specific functional characteristic. In addition, several are based on theories of the biological process(es) responsible for aging.

Two facts—that DR reduces the body fat content of rodents and that excess body fat is associated with the premature death of humans—led to the hypothesis that DR extends the length of life of rodents by reducing body fat content (Berg & Simms, 1960). This thinking lost favor when it was found that the life-extending action of DR can be dissociated from its effect on body fat content in rats (Bertrand *et al.*, 1980) and mice (Harrison *et al.*, 1984). However, the reduced body fat hypothesis has been "revisited" because research in the past 5 years has shown that adipose tissue secretes peptides (e.g., leptin) and cytokines (e.g., tumor necrosis factor) with powerful systemic actions that are capable of causing aging if present in excess (Barzilai & Gupta, 1999). By reducing fat content, it is postulated that DR would decrease the secretion of many of these peptides and, in this way, slow aging. Indeed, DR does decrease plasma leptin levels, presumably by reducing body fat; this finding led Shimokawa and Higami (1999) to hypothesize that the decreased level of this neuroendocrine modulator plays a key role in the antiaging action of DR. Certainly further study is warranted on the association between longevity and total body fat mass as well as the mass of specific fat depots, especially now that noninvasive imaging technology is available. However, until this association is established, the "revisiting" of the reduced body fat hypothesis merely provides a list of potential humoral mechanisms for a phenomenon that may not exist.

As discussed earlier, DR enhances apoptosis. It has been hypothesized that enhanced apoptosis underlies both the antiaging and antitumorigenesis action of DR (Warner *et al.*, 1995). Presumably, the efficient elimination of damaged, and thus probably dysfunctional, cells retards aging, and the elimination of precancerous cells would reduce the occurrence of cancer. However, as of now, there is little empirical evidence to support this hypothesis.

DR decreases body temperature in both mice and rats, though much more markedly in mice than in rats (Weindruch *et al.*, 1995). On the basis of the concept that decreasing body temperature reduces the damage inherent in living processes, it has been postulated that the antiaging action of DR may be due, at least in part, to the reduction in body temperature. However, several lines of evidence indicate that the reduction in body temperature plays, if any, a minor role in the antiaging action. First, DR increases longevity to a similar extent in mice and rats, even though the decrease in body temperature is much greater in mice. Second, although lowering the body

temperature of fish extends life span, DR can extend the life span of fish without lowering body temperature, and these two effects are additive (Walford, 1983). Finally, most of the actions of DR occur even in mice maintained in a thermal environment in which DR does not cause a decrease in body temperature (Koizumi *et al.*, 1996). Although the extension of the length of life of the C57BL/6 strain of mouse by DR does not occur in such a thermal environment, this effect was found to be due to the loss in the ability of DR at that ambient temperature to prevent lymphoma development in this lymphoma-prone strain rather than to a loss in the ability to retard aging in general. However, some physiological responses induced by DR do not occur if the rodents are maintained at an environmental temperature that prevents a fall in body temperature (Weindruch *et al*, 1995). Also, lowering of the ambient temperature of several poikilothermic species has been found to to increase life span (Finch, 1990). Therefore, it seems apparent that one must keep an open mind regarding a possible role for a decrease in body temperature in the antiaging action of DR, although as of now, it seems unlikely that it plays a major role.

DR increases the physical activity of rodents (McCarter, 2000). Because exercise in humans is known to counter many of the effects of aging, the antiaging action of DR may be due, in part, to this effect on physical activity. However, a careful investigation of this possibilty has indicated that increased physical activity probably is not an important factor in the antiaging action of DR in rodents McCarter *et al.*, 1997).

Currently there are three hypotheses of the antiaging action of DR that appear to be promising; each is based on proximate biological processes that may underlie aging. These hypotheses are worthy of detailed consideration.

2. *Oxidative Damage Attenuation Hypothesis*

Molecular oxidative damage is due to the interaction of reactive oxygen molecules, such as the hydroxyl and superoxide radicals, with cellular lipids, proteins, and nucleic acids. These reactive oxygen molecules are generated by intrinsic living processes as well as by environmental interactions. The phenomenon is referred to as oxidative stress, and it has been hypothesized that DR retards aging by attenuating oxidative stress (Sohal & Weindruch, 1996). There is much evidence that DR does, indeed, retard the age-associated cellular accumulation of oxidatively damaged molecules (Yu, 1996).

DR suppresses the age-associated increase in the rate of exhalation of ethane and pentane by rats, indicating its attenuation of lipid peroxidation (Matsuo *et al.*, 1993). Also, DR has been shown to slow the increase with age in lipid peroxidation in the kidney, liver, and brain of rats (Cook & Yu, 1998). In what is probably a related action, DR decreases the loss in fluidity of biological membranes with advancing age (Pieri, 1997; Yu *et al.*, 1992).

DR also decreases the age-associated increase in oxidatively damaged proteins in rats (Aksenova *et al.*, 1998; Youngman *et al.*, 1992) and mice (Dubey *et al.*, 1996; Sohal *et al.*, 1994b). Specifically, it attenuates the increase with age in the carbonylation of proteins and also decreases the age-associated loss of sulfhydryl groups of proteins.

DR blunts the age-associated increase in the ratio of 8-hydroxy-2′-deoxyguanosine residues to 2′-deoxyguanosine residues in rats, an indication that it decreases the accumulation of oxidatively damaged DNA (Chung *et al.*, 1992). DR also protects mice from the age-associated accumulation of oxidatively damaged DNA (Sohal *et al.*, 1994a).

This attenuation of the age-associated increase in oxidatively damaged molecules means that DR either (1) decreases the rate of generation of reactive oxygen molecules or (2) increases the effectiveness of protective and repair processes, including removal of damaged molecules and their replacement by biosynthesis. Indeed, a combination of these processes may be involved.

It is often stated that hypometabolism is the mechanism by which DR retards the aging processes. This makes sense because reactive oxygen generation would be expected to decrease in hypometabolic states, and there may well be circumstances in which DR lowers the metabolic rate. However, in both rats and mice, DR shows a marked antiaging action without lowering of the rate of metabolism per unit of lean body mass (Duffy *et al.*, 1991; McCarter & Palmer, 1992). In a report by Greenberg and Boozer (2000), the method of using lean body mass to normalize for body size is cited as the reason DR has been reported not to decrease the metabolic rate. The authors proposed that normalizing for the combined weight of the heart, kidneys, brain, and liver provides more reliable findings. When the authors did this with 22-month-old male F344 rats, it was found that DR did not lower the metabolic rate. They hypothesize that they would have found DR to decrease the metabolic rate if they had studied 6-to 18-month-old rats. Apparently, these authors feel they know the correct answer and now seek the correct model to prove it.

The unsubstantiated claim that DR retards aging by causing hypometabolism has an unfortunate effect. This is well-illustrated by a recent hypothesis on the mechanism by which DR retards aging (Imai *et al.*, 2000). This hypothesis proposes that the DR-induced reduction in metabolic rate decreases the availability of NAD, which causes an up-regulation of the deacylation activity of Sir2 proteins and chromatin silencing; it is further postulated that the persistence of genomic silencing slows aging-related processes. Apparently, the proponents of this hypothesis were unaware of the studies showing that DR can retard aging without decreasing the metabolic rate. Alas, both the lay and the scientific press published stories based on the Imai *et al.* (2000) hypothesis that, worse yet, suggested that the way DR increases life span had been uncovered! Clearly, the claim that the antiaging action of DR is due to hypometabolism is misleading and should be avoided.

Moreover, there need not be a decrease in metabolic rate to decrease the generation of reactive oxygen species. Indeed, DR has been found to decrease the rate of mitochondrial generation of superoxide radicals and hydrogen peroxide in the brain, kidney, and heart of mice (Sohal & Dubey, 1994). DR also decreases cyclooxygenase expression and cyclooxygenase-derived reactive oxygen species (Chung *et al.*, 1999).

The enhancement of antioxidant defenses is another way that DR could decrease molecular oxidative damage (Yu *et al.*, 1989). Early reports on antioxidant enzymes supported this possibility (Koizumi *et al.*, 1987; Mote *et al.*, 1991; Rao *et al.*, 1990). However, subsequent studies have not demonstrated DR's consistent enhancement of these enzymes (Gong *et al.*, 1997; Rojas *et al.*, 1993; Sohal *et al.*, 1994b). In fact, DR has been found to attenuate the age-associated increase in rat skeletal muscle antioxidant enzyme activities (Luhtala *et al.*, 1994). This report appears to contradict the finding that DR forestalls the age-associated decrease in the level of liver catalase and superoxide dismutase mRNA (Van Remmen *et al.*, 1995). The apparent discrepancy may be due to differences between muscle and liver or due to the fact that mRNA levels were measured

in liver whereas enzyme activities were assessed in skeletal muscle.

Clearly, the effect of DR on antioxidant defenses is complex and requires further study to reconcile apparently conflicting findings. Recent studies provide a good start. Cellular glutathione levels decrease with age but DR maintains youthful levels, particularly of mitochondrial glutathione, into advanced ages, and this may provide a major defense against oxidative damage (Armeni *et al.*, 1998). Also, DR blunts the age-associated increase in the iron content of kidney, brain, and liver, an effect that may well attenuate lipid peroxidation (Cook & Yu, 1998). In addition, long-term DR has been found to protect mouse lens epithelial cells from damage due to exposure to H_2O_2 (Li *et al.*, 1998) and to protect mitochondrial gene transcription against the damage of peroxyl radicals (Kristal & Yu, 1998).

As discussed earlier, DR increases repair processes. It enhances the repair of oxidatively damaged DNA. It also increases the removal of damaged proteins by proteolysis and their replacement with newly synthesized proteins (Van Remmen *et al.*, 1995).

In summary, the evidence that DR protects against oxidative molecular damage is overwhelming; moreover, the processes underlying this protection are understood at least in broad outline. Is this protection the primary mechanism responsible for the antiaging action of DR? To answer this question requires that another question be addressed. Is oxidative stress a major factor responsible for aging in rodents? Whereas many would be inclined to answer this question in the affirmative, clear evidence that such is the case is not yet at hand.

3. *Glucose–Insulin Hypothesis*

Hyperglycemia and hyperinsulinemia are known to cause aging-like damage (LevRan, 1998; Parr, 1996; Reaven, 1989). Dietary restricted rats have been found to sustain lower plasma glucose concentrations and markedly lower plasma insulin concentrations than rats fed *ad libitum*, although they utilize glucose fuel at the same rate per gram of body mass to the 3/4 power (Masoro *et al.*, 1992). These findings led to the glucose–insulin hypothesis, that is, maintenance of reduced levels of plasma glucose and insulin without compromising glucose fuel use is, at least in part, responsible for the antiaging action of DR (Masoro, 1996).

Further, it appears that the major factor in the ability of DR rodents to sustain glucose fuel use at reduced plasma glucose and insulin levels relates to enhanced insulin-stimulated glucose transport in the skeletal muscles (Cartee *et al.*, 1994). The glucose transporter GLUT–4 accounts for just about all of the insulin-stimulated glucose transport in muscle. Although DR does not change the level of skeletal muscle GLUT–4, it increases the fraction of the GLUT–4 located at the plasma membrane of the muscle cell during insulin stimulation (Dean *et al.*, 1998a). The reduction in plasma insulin during DR is attributable in large part to decreased insulin secretion by the β-cells of the pancreatic islets (Dean *et al.*, 1998b).

Whereas there has been good progress in defining the mechanisms by which DR decreases plasma glucose and insulin levels while maintaining glucose fuel use, scant evidence has accrued that directly supports the glucose–insulin hypothesis. Specifically, there is little evidence for the retardation of aging because of the sustained reduction in glycemia and insulinemia below that of normoglycemic and normoinsulinemic levels. By decreasing glycation and glycoxidation, lower plasma glucose levels could slow aging. DR has been reported to decrease the formation of advanced glycation end-products in rats (Cefalu *et al.*, 1995;

Reiser, 1994). Another study found that DR decreased the age-associated accumulation of these glycation products in skin collagen, but not aortic collagen (Novelli *et al.*, 1998). It is clear that further research aimed at testing the glucose–insulin hypothesis is needed.

4. *Hormesis Hypothesis*

Although the oxidative stress attenuation hypothesis and the glucose–insulin hypothesis probably define important aspects of the antiaging action of DR, they may be too tightly focused. The hormesis hypothesis broadens the scope to include protection from a spectrum of damage (Masoro, 1998). In this discussion, hormesis is defined as follows: *Hormesis is the beneficial action(s) resulting from the response of an organism to a low-intensity stressor.*

The first question that needs to be considered is whether DR, as used in experimental gerontology, is a low-intensity stressor. The best evidence that it is a low-intensity stressor can be seen in its effects on the glucocorticoid system. Rats on DR show a modest, but significant, daily elevation in the afternoon peak concentration of plasma-free corticosterone (Sabatino *et al.*, 1991).

Is there evidence that DR has beneficial actions in addition to its antiaging action? Altough there is no evidence available in regard to long-term moderate-intensity stressors, there is abundant evidence that DR protects rodents of all ages from damage due to intense, acute stressors. The following provide examples of the scope of this protection. The acute weight loss in rats following a surgical procedure is reduced markedly by DR (Masoro, 1998). DR attenuates the inflammatory response resulting from injection of carageenan into the foot pad of young mice (Klebanov *et al.*, 1995). The ability of rats to survive a sudden marked increase in environmental temperature is enhanced greatly by DR (Heydari *et al.*, 1993). DR protects rodents from the acute damaging action of toxic drugs (Duffy *et al.*, 1995).

Does DR's increased ability to cope with acute, intense stressors have anything to do with DR's antiaging action? There are two reasons for an affirmative answer. First, genetic manipulations of fruit flies, nematodes, and yeast have yielded genotypes with extended life spans and, thus, presumably a decreased rate of aging; at all ages, these genotypes showed an increased ability to cope with intense stressors (Martin *et al.*, 1996). Second, in all species, the major cause of aging appears to be unrepaired damage due to long-term intrinsic and environmental damaging processes or agents. Thus, hormesis, the increased ability to cope with damaging processes and agents, is likely to be the major factor in the antiaging action of DR.

What are the proximate mechanisms that might underlie the protective actions of hormesis? A spectrum of protective and repair processes probably is involved. Stress response genes are likely candidates. Indeed, DR has been found to enhance the expression of heat-shock proteins in response to stress (Aly *et al.*, 1994; Heydari *et al.*, 1993, 1995; Moore *et al.*, 1998).

Another likely proximate mechanism is the hypothalamic–hypophyseal–adrenal cortical glucocorticoid system, which is known to play an important role in the ability of vertebrates to cope with damage (Munck *et al.*, 1984). Rats undergoing DR maintain elevated maximal daily plasma concentrations of free glucocorticoid throughout the life span (Sabatino *et al.*, 1991). It has been proposed that the elevated levels of glucocorticoid enable the rodent to better cope with the daily insults that cause aging, including damage secondary to the responses of primary defense systems, such as immune and inflammatory

reactions. Indeed, it has been shown that the ability of DR to protect against chemically induced tumors is lost if the mice are adrenalectomized (Pashko & Schwartz, 1992).

Clearly, many of the actions of DR, including the antiaging action, are within the realm of hormesis. However, further work is needed to fully define the proximate mechanisms by which hormesis retards the aging processes.

D. Evolution of the Antiaging Actions of Dietary Restriction

Holliday (1989) hypothesized that the antiaging action of DR evolved in nature in response to periods of scant food supply. Specifically, he proposed a survival advantage for those animals that have genomes enabling them to respond to food shortage by diverting energy from reproduction to somatic maintenance. He further postulated that, when there is a sustained reduction in the availability of food (e.g., DR in an experimental setting), the diversion of energy to maintenance continues; in line with the disposable soma theory, this slows the aging processes. Masoro and Austad (1996) expanded this view, emphasizing the role of unpredictable periods of food shortages and the importance for survival of the diversion of energy to cope with environmental challenges. The expanded version of the evolution of the antiaging action of DR embraces both the disposable soma theory and the hormesis concept.

Walford and Spindler (1997) agree that the antiaging action of DR derives from the evolutionary adaptation to food shortage. Based on metabolic characteristics, they feel the antiaging action is one of a family of such adaptations, which includes hibernation.

Hart and Turturro (1998) proposed an evolutionary concept of the antiaging action of DR similar to that of Holliday, in which they too utilize, without so stating, the disposable soma theory of aging. The only new contributions from their proposal are the speculations about the cellular and metabolic bases of the antiaging action.

III. Nonhuman Primate Models

Does DR retard aging in humans? This has been debated for a long time. Unfortunately, it is not possible to provide a definitive answer because a carefully designed and executed investigation with human subjects has not been done, nor is it likely such a study will ever be done. In lieu of this, nonhuman primates are being studied in this regard, for if DR retards aging in these animal models, it is likely to do so in humans.

A. Ongoing Studies

There are three major ongoing studies of DR in rhesus monkeys. One is an intramural study conducted by the National Institute on Aging (NIA) (Ingram *et al.*, 1990). In this NIA study of male and female monkeys, DR refers to food intake 30% below that of a control group. DR was initiated at 1–2 years of age, 3–5 years of age, or greater than 15 years of age. (The maximum life span of the rhesus monkey is estimated at 40 years.) Most of the monkeys are utilized in long-term, longitudinal studies, but some are assigned to short-term, cross-sectional studies.

Another study on male and female rhesus monkeys is being carried out at the University of Wisconsin (UW) (Kemnitz *et al.*, 1993). In the UW Study, DR was initiated at 8–14 years of age, but the 30% reduction in food intake is 30% below that of the intake of the same animal during the period just prior to the initiation of DR. Most of the monkeys in this program are used in long-term, longitudinal studies, but some are assigned to short-term, cross-sectional studies.

The third study, the UM study, emerged from ongoing obesity and diabetes mellitus research at the University of Maryland (Hansen & Bodkin, 1993). In this long-term, longitudinal design, DR was initiated in male rhesus monkeys at 11–12 years of age. The level of restriction is that needed to maintain a stable body weight of 10–12 kg. Thus, in this study, the control monkeys are allowed to become obese with increasing age. Indeed, the food intake of the DR monkeys in the UM study is such that their body weight and composition are similar to those of the control group of the NIA study. Therefore, the UM study differs from the NIA study and the UW study in regard to the level of food intake of the DR monkeys, and this difference must be kept in mind when comparing the results of the studies.

B. Dietary Restriction and Body Composition

In the UW study, body composition is assessed semiannually. The results of 7.5 years of such assessment have been reported (Colman *et al.*, 1999). DR resulted in less total body fat and a lower percentage of body fat in the abdominal region. In addition, the animals had a sustained reduction in plasma leptin levels. The DR monkeys also sustained a small, but significantly lower lean body mass than the control group. DR was found to have similar effects on total fat mass and lean body mass in the NIA study (Lane *et al.*, 1997b). As might be expected from the study design, the rhesus monkeys on DR in the UM study have less body fat than the control monkeys (Hansen & Bodkin, 1993).

C. Dietary Restriction and Physiological Characteristics

No attempt will be made here to present all the changes that DR has been found to produce in nonhuman primates. Rather, those changes will be discussed that may indicate a possible antiaging action based on the rodent studies or on current views of the biological nature of mammalian aging.

The effect on metabolic rate is a good starting point. In the NIA Study, metabolic rate per kilogram of lean body mass declined upon initiation of DR, but rose to that of control animals during long-term DR (Lane *et al.*, 1995a). In the UW Study, DR was reported to decrease metabolic rate per kilogram of lean body mass during the first 30 months (Ramsey *et al.*, 1997), but by 42 months it was no longer decreased (Ramsey *et al.*, 1996). In the UM study, the metabolic rate per kilogram of lean body mass remained decreased even after 10 years of DR (DeLaney *et al.*, 1999).

In the NIA study, the influence of DR on motor activity was not consistent, with only one of the two groups of monkeys studied exhibiting an increase in activity (Weed *et al.*, 1997). In the UM Study, DR increased motor activity compared to age-matched controls; however, if activity was adjusted for differences in body size, DR had no effect on motor activity (DeLaney *et al*, 1999).

In the NIA study, body temperature was decreased by DR (Lane *et al.*, 1996). No such effect on body temperature was observed in the UM study (DeLaney *et al.*, 1999).

In the UW study, long-term DR decreased fasting plasma glucose and insulin levels and increased insulin sensitivity (Kemnitz *et al.*, 1994). The increase in insulin action was not associated with a change in the abundance of GLUT4 glucose transporter, phosphatidylinositol 3-kinase, or insulin receptor substrate (Gazdag *et al.*, 2000). DR also lowered fasting plasma glucose and insulin levels in the monkeys of the NIA study (Lane *et al.*, 1995b). In the UM study, DR lowered fasting plasma insulin levels and

increased insulin sensitivity, but did not decrease fasting plasma glucose levels Bodkin *et al.*, 1995).

DR also influences the plasma lipoproteins of rhesus monkeys. It increases the level of the HDL_2 fraction of plasma high-density lipoproteins (Verdery *et al.*, 1997) and changes the structure of the low-density lipoproteins so as to decrease their interaction with arterial proteoglycans (Edwards *et al.*, 1998).

Whereas other data have been reported on physiological characteristics influenced by DR, the relationship of most of these findings to possible antiaging action is not evident. However, two such findings may have bearing and therefore should be mentioned. First, there is an age-associated decline in serum dehydroepiandrosterone sulfate in the rhesus monkey, and this decline is slowed by DR Lane *et al.*, 1997a). Second, DR lowers serum insulin-like growth factor-1 and growth hormone in rhesus monkeys Cocchi *et al.*, 1995).

The major physiologic responses to DR in the three long-term studies of rhesus monkeys are summarized in Table I. Again it must again be emphasized that the design of the UM study differs from that of the NIA and UW studies, in that the food intake of the DR monkeys in the UM study is considerably greater than that in the other two studies. This fact must be kept in mind when comparing the findings of the three studies.

It should also be noted that a short-term DR research program is being carried out on cynomolgus monkeys. Early findings indicate that the effects of DR on body fat and carbohydrate metabolism in this species are similar to those found with rhesus monkeys (Cefalu *et al.*, 1997).

D. Dietary Restriction and Disease Processes

Whereas a great deal of detailed histopathological data on the effects of DR on rodents has been published, such data are not yet available for nonhuman primates. However, the following risk factors for age-associated disease have been assessed: body fat mass and distribution, carbohydrate metabolism, and serum lipoproteins. On the basis of these findings, it appears likely that long-term DR will retard age-associated cardiovascular disease in rhesus monkeys. Moreover, DR prevents the development of type II diabetes in the rhesus monkey (Hansen & Bodkin, 1993). Also, in male rhesus monkeys of ages greater than 18 years, risk factors for cardiovascular disease and diabetes decreased during the first 12 months of DR (Lane *et al.*, 2000).

Table I
Summary of Salient Physiological Responses of Rhesus Monkeys to DR

Physiological characteristic	NIA study	UW study	UM study
Decreased body fat	Yes	Yes	Yes
Decreased lean body mass	Yes	Yes	—
Decreased metabolic rate:			
Short-term	Yes	Yes	Yes
Long-term	No	No	Yes
Decreased body temperature	Yes	—	No
Decreased fasting plasma glucose	Yes	Yes	No
Decreased fasting plasma insulin	Yes	Yes	Yes
Increased insulin sensitivity	—	Yes	Yes

E. Evaluation of Possible Antiaging Action

DR has a broad spectrum of physiological effects in rodents, many of which also occur in nonhuman primates. This provides some indication that DR may have an antiaging action in monkeys similar to that seen in rats and mice. However, this possibility must be tempered by the fact that, in rodents, the relationship between a particular physiological effect and the antiaging action of DR has yet to be established. On a positive note, several of the physiological modifications caused by DR in monkeys decrease known risk factors for age-associated disease. It has been suggested that the antiaging action of DR can be assessed by measuring suitable biomarkers of aging (Roth *et al.*, 1999). Unfortunately, clear evidence of the existence of one or more suitable biomarkers has yet to emerge.

A longevity study using rhesus monkeys is in the planning stage at the National Institute on Aging (Roth *et al.*, 1999). The results of such a study, including histopathological assessments combined with the physiological assessments now underway, should provide a definitive answer to the question of the antiaging action of DR in nonhuman primates. However, it must be noted that it will take 20 or more years to complete all of this work, even if the study is launched in the immediate future.

IV. Insights from Dietary Restriction Studies

The fact that DR has such a marked antiaging action in rodent models makes it an important tool for learning about the basic biological processes underlying mammalian aging. Uncovering the mechanism or mechanisms by which DR retards aging is likely to yield a database that would be invaluable in the search for human antiaging interventions.

A. Biological Processes Underlying Aging

The findings to date from DR studies provide support for three of the current concepts of the biological basis of aging. That DR protects rodents from an age-asociated increase in cellular oxidative damage adds further support for the oxidative stress theory of aging. The fact that rodents on DR regimens sustain lower plasma glucose levels and much lower plasma insulin levels lends credence to the view that glycemia and insulinemia are important factors in mammalian aging. Finally, the ability of DR to protect rodents from a range of stressors, coupled with the genetic evidence on the relation between aging and the resistance to stressors in invertebrate models, points to low-intensity, long-term stressors likely playing a major role in aging.

B. Potential Antiaging Interventions

Even if long-term DR were demonstrated conclusively to have a strong antiaging action in humans, it is not likely that many would make use of it. However, understanding the mechanism(s) underlying the antiaging action of DR in rodents could well yield insights on the kinds of specific interventions likely to retard aging in many mammalian species, including humans. The development of pharmacological agents with antiaging actions similar to those of DR is a particularly promising possibility once the basic mechanism(s) is (are) known.

V. Future Research Directions

The future direction of research on DR in nonhuman primates seems to be

outlined clearly. The goal should be to establish, without equivocation, that DR does slow the aging processes in the rhesus monkey and to do so requires determination of its effects on longevity and on the occurrence and progression of age-associated diseases. These are daunting but doable studies.

The rodent model should continue to be used in searching for the mechanism(s) underlying the antiaging action of DR. Transgenic models that overexpress or underexpress specific genes are one approach that is being pursued actively. For such findings to be interpretable, manipulations must alter one or more characteristics in a fashion similar to DR. If the antiaging action of DR is due to the alteration of a specific characteristic, e.g., decreased plasma glucose concentration, then such an approach can yield easily interpretable results. However, because the antiaging action of DR may well be due to the simultaneous alteration of several characteristics, there is a good chance that transgenic technology may provide little insight. Moreover, it is technically challenging to establish that a transgenic model is altered only in regard to the DR characteristic(s) being assessed.

Almost the opposite approach is provided by gene expression profile methodology, in which a broad array of genes is assessed. This approach enables the identification of patterns of genes that change with age and dietary restriction. However, this approach does have pitfalls, as revealed by a report on the effect of DR on gene expression in skeletal muscles of mice (Lee *et al.*, 1999). Of the 6347 genes monitored, the expression of 113 changed with age. Most of these changes were completely or partially prevented by DR. This many genes being affected by DR, even when only a small fraction (5–10%) of the genome is being assessed, presents the investigators with an interpretational dilemma. The authors of this paper tried to make sense of the findings by interpreting the data in terms of alterations of genes expressing enzymes involved in energy metabolism. However, this interpretation is based on a preconceived view of the mechanism of the antiaging action of DR, an approach replete with hazards. Another problem made evident by the report of this study is the need to verify the results of this technology by another independent method; the authors did not do this and, indeed, to do so involves a substantial amount of additional work.

Thus, future research on mechanisms of the antiaging action of DR will not be easy. However, careful, thoughtful application of these new methodologies as well as others that are sure to emerge should bear fruit in the long run.

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