Beneficial Biochemical Outcomes of Late-Onset Dietary Restriction in Rodents

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ABSTRACT: Dietary restriction (DR) or caloric restriction (CR) is the well-established means to retard aging, leading to prolongation of mean and maximum life span in many animal models. We have been interested in the possibility of extending the span of health of elderly people rather than increasing longevity, and therefore studied the effects of DR/CR initiated late in life in rodent models. We restricted food for 2–3.5 months in mice or rats of middle or old ages, which would perhaps be equivalent to 50–70 years of age in humans. We found that: (1) Potentially harmful altered proteins were reduced in the animals' tissues. (2) Extended half-life of protein in aged animals was shortened in mouse hepatocytes, suggesting improved protein turnover. (3) Reduced proteasome activity was upregulated in rat liver and skeletal muscle. (4) Protein carbonyls were decreased in rat liver mitochondria and skeletal muscle cytoplasm, and also oxidative DNA damage was reduced in rat liver nucleus, suggesting amelioration of oxidative stress. (5) Reduced apo A-IV and C-III metabolism in aged mouse was restored, suggesting increase in reduced fatty acid mobilization. (6) The carbonyl modification in histones that was paradoxically reduced in aged rat was increased to the level of a young animal, suggesting restoration of reduced transcription. These findings in rodents suggest a possibility that DR/CR is beneficial if applied in middle-aged or early senescent obese people. We argue, however, that application of late life DR/CR can be harmful if practiced in people who are already eating modestly.

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INTRODUCTION

In industrialized countries where longevity has been realized by a large portion of the elderly population, there is a tendency not to wish to live longer but to extend the span of health and a healthy life. Various means have been attempted to prolong the span of health including antioxidant supplementation, hormone replacement therapy, exercise and dietary restriction (DR) or caloric restriction (CR). No robust means other than DR or CR have ever been reported, which can improve a variety of age-related impairments and pathologies. DR/CR is the most well-documented way to retard aging in many animal models.[1] In recent years the effects of CR on the yeast model have attracted considerable interest because it may generalize the life-prolonging effect of DR/CR from single-cell eukaryotic organisms to mammals.[2] However, it should be borne in mind that the definition of aging is quite different between models for yeast and multicellular models, such as nematodes, fruit flies, and rodents. While the age of yeast is mostly measured by the number of replications or shedding of progenitor cells from a mother cell, that of multicellular organisms is usually measured by the elapsed time after birth or in the adult stage after eclosion in the case of insects. Therefore, one must be cautious in making generalizations in models as disparate as yeast and multicellular organisms that are apparently similar but unlikely to be homologous.

It is noted that CR/DR in rodents not only extends life span but also retards physiological decline with age and delays the onset of age-related diseases; more importantly, they can partially reverse changes that occur with advancing age when applied later in life. We summarize here our findings on the influence of later life DR on biochemical parameters in rodents.

BRIEF OVERVIEW OF ANTIAGING EFFECTS OF LIFELONG CR/DR

Since the seminal publication of McCay that reported dietary restricted rats live longer than *ad libitum* fed animals, numerous studies have been reported on this interesting phenomenon. It is now well established that mean and maximum life span of rodents are 30–50% longer if calories are restricted by 30–40% for life after weaning or young ages compared with *ad libitum* fed animals.[3] A variety of biochemical and physiological age-related changes are retarded including decline of induction of heat shock proteins in hepatocytes,[4] decrease in the number of dopamine receptors,[5] expression of the NMDA receptor in the striatum,[6] and so on. Age-related pathologies, such as spontaneous cancer

incidence[7] and renal disorders,[8] are also retarded. Interestingly, short-lived animals with genetic disorders can live longer by CR with reduced manifestation of the disease. For example, the life span of spontaneous hypertensive rats with genetic defect becomes nearly as long as wild-type animals with reduced cardiac and renal lesions,[9] average life span being 30 months of age as compared with 18 months in *ad libitum* fed animals. In senescence-accelerated mice with apparent genetic defect(s) the age-associated immunological decline is reported to be delayed.[10] It is thus remarkable that restriction of calories can improve even genetic defects.

INTERVENTION OF AGING BY CR/DR INITIATED IN MIDDLE OR OLD AGES

A majority of studies on the effects of CR/DR in aging rodents have used lifelong regimens as described above probably because significant extension of life makes them more interesting. Reports on DR/CR initiated later in life are much more limited. Weindruch *et al*. have reported that the mean life span of mice whose food was restricted from 12 to 13 months of age is extended by 10–20% with reduced incidence of cancer, although these effects were modest compared with those by lifelong CR.[11] They also found that age-related decline of immunological activity of splenocytes in response to PHA stimulus was attenuated by CR from 22 months of age.[12] Means *et al*. reported that mice subjected to CR (60 % of *ad libitum* level) from 14 to 25 months of age had better cognitive functions.[13] Lipman *et al*., however, found that rats fed 70% of *ad libitum* level of food from either 18 or 26 months of age until death did not show significant difference in mean life span, carcinogenesis or pathologies from *ad libitum* fed animals.[14] More recently, CR regimen in rats during 1 year starting at 24 months of age was reported to reduce brain mitochondrial H_2O_2 production by 24% and oxidative damage to the mitochondrial DNA by 23% compared with unrestricted counterparts.[15] Spindler's group has reported that 8 weeks of CR initiated at 19 months of age reduced incidence of liver and other tumors, resulting in a significant average and maximum life span extension in mice.[16] They also reported that the regimen caused similar changes of hepatic gene expression as seen in lifelong CR.[17] All these findings suggest that DR initiated even late in life can restore youthful cellular functions, thus possibly promoting quality of life.

If we are to translate outcomes of animal studies to human, it would obviously not be realistic to recommend starting DR/CR from childhood or adolescence, even if such lifestyle would prove to be beneficial later in life. Studies on late-life CR in rodents could potentially provide the basis for human application for better quality of life although life span extension would not be remarkable, if any. We have, therefore, conducted studies on late-life DR in mice and rats focusing on age-related changes, such as protein oxidation/degradation,

DNA damage/repair, and lipid metabolism, starting the regimen in the middle or old ages that are presumed to be equivalent to 50–70 years of age in human.

AGE-RELATED INCREASE IN ALTERED PROTEIN AND DECREASE BY DR IN AGED RATS AND MICE

Altered proteins as judged by reduced heat stability, molecular activity, and posttranslational modifications increase with age. Such proteins cannot only be useless but also gain harmful functions, thus possibly promoting aging. Heat-labile forms of aminoacyl tRNA synthases increase with age in various tissues of mice and rats.[18] We found that the percentage of heat-labile enzymes in the liver and brain of old mice (23.5-month old) was reduced to the levels of young adults (11-month old) within 2 months of DR (60% of *ad libitum* level).[19] The finding suggests that the degradation of altered proteins was promoted in these tissues by DR. It was noted that the effect was more evident in the liver within 1 month while it took more time to be effective in the brain, suggesting that cell turnover might also be involved in addition to molecular turnover in reducing the altered proteins. Generation of carbonyl moieties has been used as a marker of oxidative alterations of proteins although it is controversial whether protein carbonyls increase with age or not.[20,21] We found that the carbonylation of mitochondrial proteins from livers of old rats (30-month old) is reduced to the level of *ad libitum* fed young adults (10-month old) by 3.5 months of every-other-day feeding, which resulted in about 35% decrease in body weight compared with *ad libitum* fed age-matched animals.[22] This result is consistent with the report that the generation of reactive oxygen species (ROS) is reduced by lifelong CR, ameliorating oxidative damage to DNA[23] and membrane lipids.[24] These findings in mice and rats suggest that oxidative damage increased with age can be reduced by relatively short-term DR initiated in old age.

EFFECT OF DR ON PROTEIN TURNOVER AND PROTEASOME ACTIVITY IN OLD AGES IN MICE AND RATS

The reduction of altered proteins by DR suggests that degradation of such proteins might be upregulated in the DR animals. We have studied this issue by measuring half-lives of proteins introduced into primary cultured mouse hepatocytes and those endogenously pulse-labeled with a radioactive amino acid.[25] Half-lives of horseradish peroxidase, ovalbumin, egg white lysozyme, and endogenous proteins were extended by about 1.5-fold in the cells from old animals (23-month old) compared with those from young counterparts (3- to 6-month old), but those in old animals became as short as in the young ones

after 2 months of DR.[26] These results suggest that the reduction of altered proteins described in the previous section can be explained at least in part by the increased turnover of proteins.

The degradation of intracellular proteins is generally catalyzed by the lysosomal and/or nonlysosomal pathways in which the major pathway consists of an ubiquitin/proteasome system that is involved in the degradation of not only damaged proteins but also regulatory proteins, such as transcription factors and cell cycle regulators, required only temporarily during cellular processes.[27] The degradation of the proteins introduced into the hepatocytes was inhibited by a proteasome inhibitor while lysosome inhibitors, such as ammonium chloride, had no effect, suggesting that the decreased activity of proteasomes is responsible for the reduced turnover of proteins with age (our unpublished results). We have looked at the age-related changes of the proteasome activity in rat livers. Activities of both 26S and 20S forms of the enzymes separated on glycerol gradient centrifugation were significantly reduced with age.[28] The total activities were upregulated by 3.5 months of DR in old rats (26.5-month old), restoring them to the level of young animals (10-month old). Interestingly, these changes in activities with age and DR were not accompanied by change in the amount of enzyme protein as measured by antibodies against subunits of the enzymes[29] (our unpublished results). It is therefore likely that the quality rather than the quantity was changed by age and DR. It is conceivable that the proteasomes themselves are altered with age causing reduced turnover of proteins and that DR can "rejuvenate" the enzymes by replacing those that are damaged with newly synthesized intact molecules. Thus, DR even initiated late in life appears to renew proteasomes and thereby promote degradation of altered proteins.

EFFECT OF DR ON LIPOPROTEIN METABOLISM IN OLD AGES IN MICE

The regulation of triglyceride and lipoprotein metabolisms is important in preventing atherosclerosis and other age-related vascular diseases.[30] The storage of triglyceride in adipose tissues and its mobilization for use in other tissues are dependent on plasma lipoproteins. The effects of late-onset DR on the plasma lipoproteins and their mRNA were studied in the liver of fasting mice. We have shown that apolipoprotein A-IV (apo A-IV) in plasma and its mRNA in the liver increased remarkably following 2 to 3 days of fasting in young mice (6-month old) but very little in old animals (25-month old).[31] The apo C-II mRNA changed similarly but apo C-III mRNA was decreased significantly by fasting. The apo A-IV is present in the chyromicron and high-density lipoprotein (HDL) in mice where it activates lipoprotein lipase (LPL) in the presence of apo C-II that promotes fatty acid mobilization.[32] The apo C-III is known to inhibit LPL. Modulation of the expression of these proteins in response to

fasting, therefore, implies that it can enhance fatty acid mobilization to meet the energy demand when food supply is limited. In old mice; however, apo A-IV was induced little and apo C-III mRNA did not decrease significantly by fasting, which suggests that fatty acid mobilization is impaired. When the 22-month old mice were subjected to DR for 3 months resulting in a body weight reduction of 30% compared with *ad libitum* fed counterparts, the plasma apo A-IV and its mRNA induced in the liver of the fasted old DR animals were higher than in *ad libitum* fed 25-month old animals subjected to fasting.[33] The level of apo C-III mRNA in the DR animals was reduced by fasting. These results suggest that DR can shift triglyceride metabolism to higher fatty acid mobilization in response to fasting by upregulating LPL activity and probably also in nonfasting conditions. It is therefore conceivable that late-life DR may help reduce the risk of vascular diseases, such as atherosclerosis, enhancing lipid metabolism.

EFFECT OF LATE-ONSET DR ON OXIDATIVE STRESS ON THE SKELETAL MUSCLE AND TENDON IN RATS

Aging of mammals is associated with decline in muscle mass (sarcopenia) and force generation.[34,35] Fall and bone fracture are serious problems in elderly people and are in part due to muscle weakness. Force generated in the sarcomere in the muscle is transmitted to bones for locomotion in which the tendon plays an essential role. Age-related changes in the antioxidant system and oxidative damage to proteins of the skeletal muscle have been reported.[36,37] The tendon, however, has remained unexplored in this respect. In terms of effects of DR, particularly DR initiated late in life, limited information is available on the skeletal muscle, and the same is true of the tendon. We therefore studied the effect of late-onset DR on oxidative status in the gastrocnemius muscle and Achilles tendon in rats.[38] The activities of antioxidant enzymes per unit amount of protein were much lower in the tendon than in the skeletal muscle. The cytoplasmic Cu, Zn-superoxide dismutase (Cu, Zn-SOD) activity, and its protein content increased significantly with age, but the increase was attenuated or reversed by 3.5 months of DR in old animals (30-month old) in both the skeletal muscle and tendon compared with adult animals (20-month old). On the other hand, the mitochondrial Mn-SOD activity and its content increased in the skeletal muscle with age although it did not change significantly in the tendon. The DR reduced the age-related increase of the Mn-SOD content in the skeletal muscle, however, no significant change was observed in content in the tendon by DR. The activities of glutathione peroxidase and catalase were also increased with age significantly and this change was eliminated by DR in both tissues. While protein carbonyls did not change significantly with age or by DR in the skeletal muscle, a nearly twofold increase was observed in the tendon in old animals compared with the adults; the DR reduced the level by

50%. The trypsin-like activity of proteasome declined by 45% with age in the skeletal muscle, and the DR restored the activity to a level comparable to that of adult animals. In the tendon, both chymotrypsin- and trypsin-like activities of the proteasome declined markedly (50–60%) with age. The activities tended to be higher in the DR animals but statistically not significantly different from sedentary controls. These findings suggest that DR may improve locomotive functions in old animals by reducing oxidative stress in the tendon.

CARBONYL MODIFICATION OF RAT LIVER HISTONES: EFFECTS OF AGE AND LATE-LIFE DR

The carbonyl modifications have been used to evaluate oxidative status of proteins as described in previous sections. Generally, protein carbonyls are reported to increase with age in soluble and mitochondrial fractions, thus possibly causing functional decline of cells.[39,40] We were interested in age-related changes of carbonylation of histones. There was only one report on histone carbonylation when we began the study but none with respect to aging.[41] Histones are highly basic proteins that are essential for chromatin structure and functions in eukaryotic cells. The posttranslational modifications of histones, such as acetylation, methylation, phosphorylation and ADP-ribosylation that influence the interaction with DNA, can modulate gene expression, replication, and DNA repair, attracting considerable interest as epigenetic changes that occur in development, differentiation and aging as well as diseases, such as cancer.[42,43] We found that histones except H4 are carbonylated *in vivo* to variable extents, H1 and H2A/2B being more carbonylated than H3[44]; it seems that histones H3 and H4 in the inner core of nucleosomes are more resistant to the modification. *In vitro* artificial oxidation of histones resulted in more general carbonylation of all histone types including H4, suggesting that the variable carbonylation of histones reflects the *in vivo* situation. Interestingly, we found a significantly higher carbonylation in histones in young rat livers (5-month old) compared with old counterparts (30-month old). This was unexpected because numerous studies have shown that protein carbonyls increase with age. Since carbonylation of proteins mostly occurs in basic amino acid residues,[45] the carbonylation of histones that are rich in Lys and Arg residues could mask the positive charge and thus may affect the compactness of chromatin and recruitment of transcription factors and repair enzymes by reducing their interaction with DNA. It is, therefore, possible that age-related decrease in histone carbonylation might cause reduced chromatin functions. This interpretation is analogous to that for acetylation and methylation that play a crucial role in modulating gene expression. These well-documented histone modifications are reversible but the carbonylation is not. The reduced carbonylation of histones in older animals may be due to replacement of the highly carbonylated molecules by less carbonylated ones by metabolic or cellular turnover.

After 2 months of DR with every-other-day feeding in 28-month old rats, the histone carbonylation was increased to the level close to that of young animals.[44] This is again paradoxical if we assume that the histone carbonylation is due to oxidative stress since such stress is reported to be attenuated in DR animals. The DR may thus relax chromatin structure and thereby activate chromatin functions, such as transcription and DNA repair, in the aged tissue. These findings may point to a new physiological role of protein carbonylation. Obviously, further studies on the chemical nature and biological implications of histone carbonylation are required.

EFFECT OF LATE-LIFE CR/DR ON OXIDATIVE MODIFICATION AND REPAIR OF DNA OF THE LIVER

The oxidative damage to the nuclear and mitochondrial DNA is increased with age.[46] The DNA base modifications in the nuclei can induce cancer and possibly change gene expression.[47] Oxidative damage to the mitochondrial DNA may impair energy metabolism, forming a vicious cycle of functional decline of these organelles and thereby causing aging.[48] We studied effects of DR in old rats on the amount of 8-oxo deoxyguanosine (8-oxodG) in the nuclear DNA of the liver. The DR initiated at 28 months of age reduced the amount significantly after 2 months (our unpublished results). The activity of the repair enzyme OGG1 (8-oxo guanine DNA glycosylase/AP lyase)[49] was higher in the *ad libitum* fed old animals (30-month old) than in young animals (5-month old); the DR lowered the activity. These findings suggest that the OGG1 activity was upregulated in the old animals because of the increase in oxidative damage to DNA while 2 months of DR downregulated the activity because the higher activity was no longer required after the damage was reduced.

CONCLUSIONS AND A PERSPECTIVE FOR HUMAN HEALTH

Despite the findings that CR/DR in later life does not prolong life span remarkably in rodents, it can have a variety of potentially beneficial effects, such as reducing altered proteins, increasing protein turnover, upregulating proteasome activity, improving lipid metabolism, and changing the pattern of histone carbonylation that might improve chromatin functions. Importantly, CR/DR appears to retard biological aging and be able to rejuvenate middle-aged or old animals in terms of some biological parameters that decline with age. These biological effects of late-life CR/DR seen in rodents remain to be tested in human. Reports that risks of diseases, such as atherosclerosis, are reduced by CR in the obese or nonobese population suggest that late-life

CR/DR might be effective in retarding onset of diseases and improving quality of life in human, thus extending the span of good health.[50,51] It should, however, be pointed out that CR/DR can be harmful by increasing the risk of diseases, such as osteoporosis and sarcopenia, if practiced in elderly people. This issue has been discussed elsewhere in more detail.[52,53]

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