

SURVEY AND SUMMARY

Caloric restriction and genomic stability

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ABSTRACT

Caloric restriction (CR) reduces the incidence and progression of spontaneous and induced tumors in laboratory rodents while increasing mean and maximum life spans. It has been suggested that CR extends longevity and reduces age-related pathologies by reducing the levels of DNA damage and mutations that accumulate with age. This hypothesis is attractive because the integrity of the genome is essential to a cell/organism and because it is supported by observations that both cancer and immunological defects, which increase significantly with age and are delayed by CR, are associated with changes in DNA damage and/or DNA repair. Over the last three decades, numerous laboratories have examined the effects of CR on the integrity of the genome and the ability of cells to repair DNA. The majority of studies performed indicate that the age-related increase in oxidative damage to DNA is significantly reduced by CR. Early studies suggest that CR reduces DNA damage by enhancing DNA repair. With the advent of genomic technology and our increased understanding of specific repair pathways, CR has been shown to have a significant effect on major DNA repair pathways, such as NER, BER and double-strand break repair.

INTRODUCTION

More than 70 years ago, McCay and his colleagues demonstrated that a reduction in total food intake after weaning significantly increased both mean and maximum life spans of laboratory rats (1). Over the last seven decades, numerous laboratories have successfully repeated McCay's findings using various strains of rats and mice as well as non-mammalian species, such as fish and flies (2–6). Thus, food restriction has been established as a powerful experimental tool, and the anti-aging action of

food restriction has become one of the most active areas of research in the realm of biogerontology (6). While life span extension by food restriction appears to be due to alterations in aging processes, the underlying mechanism(s) by which food restriction exerts its anti-aging effects remain elusive. Identification of important anti-aging and anti-tumor targets of food restriction and elucidating the molecular mechanisms by which food restriction exerts its beneficial effects could eventually provide targets for intervention in humans.

Extensive studies by Masoro's (7) and Good's (8) laboratories conclusively demonstrate that the increase in the survival of rodents by food restriction is due to a decreased intake of calories, i.e. caloric restriction (CR). Interestingly, the increase in survival is attained along with retardation in disease and pathology, e.g. cancer; thus, it is often proposed that CR increases the survival of rodents simply by retarding disease processes rather than altering aging and senescence (4). Although the distinction between senescence and disease processes is difficult to interpret, research over the last three decades demonstrates that CR retards the changes that occur with age in most physiological processes and that these changes generally precede any alterations observed in pathology and disease (4,8–10). While restricting the dietary intake of proteins and restriction of dietary methionine have been shown by some laboratories to increase both mean and maximum life spans of rats (11,12), other laboratories have reported that methionine restriction is not the key to life span extension by food restriction (13,14). To date, CR is the best described experimental strategy known to increase the survival of mammals by retarding aging. Thus, CR offers scientists a powerful tool for studying the biological and molecular mechanism(s) involved in aging and senescence.

Although it is well established that CR (without malnutrition) increases survival of rodents and reduces the pathology and disease associated with aging, the molecular and cellular event(s) underlying these various outcomes are currently unknown. Initially, several hypotheses were advanced to explain the mechanistic

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basis by which CR prolonged survival of laboratory rodents (4,15–19). Originally, it was proposed that CR increased survival by retarding growth and development. Later, reduction in body fat content was proposed as the physiological basis for the increased survival rates of rodents fed a CR diet (20). Subsequently, Sacher (21) proposed that CR increased survival by reducing the metabolic rate of the rodents. However, studies over the last three decades have refuted these hypotheses (22,23).

Recent efforts on dissecting the mechanism(s) by which CR extends life span lean towards the impact of CR on stress response and signaling mechanisms. Currently, the major hypotheses proposed are the oxidative damage attenuation hypothesis, the altered glucose–insulin system hypothesis, the alteration of the growth hormone-IGF-1 axis hypothesis and the hormesis hypothesis (14). In the oxidative damage attenuation hypothesis, CR has been demonstrated to reduce the accumulation of oxidative damage to biological molecules including DNA, protein and lipids in rodents (24,25). This reduction in oxidative damage is proposed to be due to a decline in the generation of reactive oxygen species (ROS) (26), an enhancement of protective mechanisms (27–31), an increase in repair capacity (32–34) or a combination of all the aforementioned. However, recent findings suggest that oxidative stress, in at least some studies, does not alter aging processes; thus, knowing whether CR extends life span via a reduction in oxidative damage remains elusive (14). The premise of the glucose–insulin hypothesis is that CR reduces the levels of plasma glucose and insulin/insulin signaling. Ultimately, CR-mediated life extension could be a result of increased glucose effectiveness, insulin responsiveness or both (35–39). This hypothesis remains exciting in light of data that show loss of the insulin signaling system results in life extension in nematodes, fruit flies

and mice (14). Moreover, CR has the potential to reduce the IGF-1 signaling, which has been observed in mammalian models with a resultant increase life span (40,41).

The hormesis hypothesis proposes a benefit to health from low-intensity stressors (14). CR may occur through a hormesis mechanism in that it could act as a mild stressor eliciting an adaptive response namely, enhanced maintenance and repair systems. CR, as part of the hormetic effect, has been found to enhance the expression of one or more stress response genes (33,42–46). In line with CR as a low-intensity stressor, it is relevant to this article to suggest that particular enzymes involved in DNA repair pathways may function as stress response genes when exposed to low intensity stress, such as CR.

Caloric restriction and genomic stability

The importance of preventing an accumulation of damage to genetic material cannot be overstated. Protection of DNA is believed to be a key element in preventing cancer as well as in delaying or preventing the aged phenotype. When DNA damage persists in the genome, through replicative processes and/or through transcription-associated mutagenesis, this damage becomes permanent, in the form of mutations and/or chromosomal breakage and instability. This increased genomic instability promotes both carcinogenic and aging processes, as outlined in Figure 1. A question that recently has been addressed by numerous laboratories is whether DNA repair capacity can be manipulated, i.e. whether key enzymes in DNA repair pathways are stress response genes and whether these enzymes could be upregulated in response to environmental stimuli, and are there environmental factors that play a role in determining the effectiveness of these critical pathways? In support of a role for the environment in modulating the efficiency of

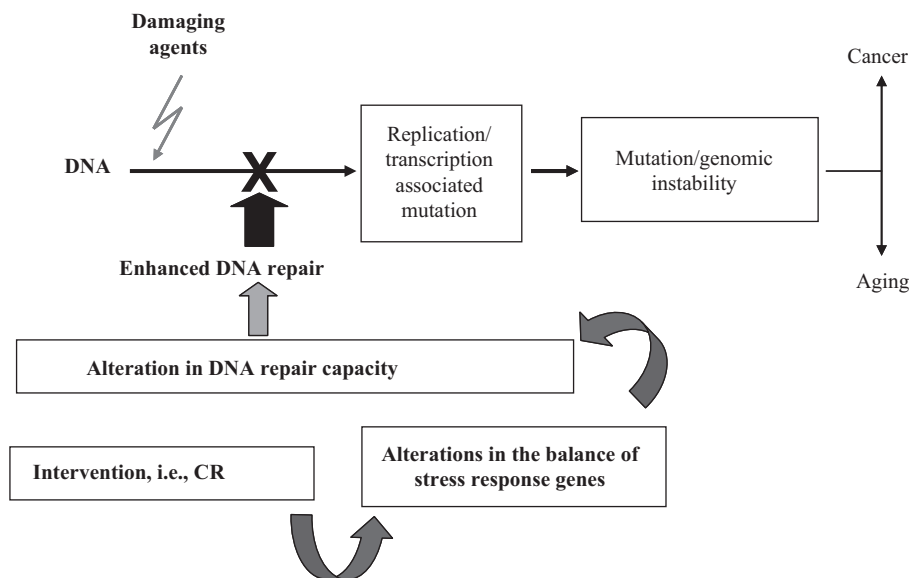


Figure 1. Modulation of genomic stability.

DNA repair capacity, numerous laboratories have addressed the question of whether CR improves genomic stability via enhancing the expression/activity of enzymes in DNA repair pathways (47). In other words, it is rational to propose that an age-related decline in the ability of a cell/organism to maintain the integrity of its genome is a fundamental mechanism underlying the aging process with CR being at the core of life extension through maintenance of genomic integrity (48). This hypothesis is attractive because the integrity of the genome is very important to a cell/organism and because DNA is constantly exposed to insults from both endogenous and exogenous factors and because DNA damage has been associated with various biological endpoints, including mutations, cancer and many of the age-associated diseases (49–53). Furthermore, DNA integrity, as maintained by a number of DNA repair systems, is essential for the survival of cells and organisms (53). The accumulation of DNA damage in somatic cells was first proposed as a basic mechanism in the aging process (54), and it was hypothesized that the accumulation of DNA damage eventually results in the inactivation of genes and cell death (54). In 1967, Alexander (55) extended this theory by proposing that the age-related accumulation of un-repaired DNA damage and subsequent mutations in cells would alter DNA replication and transcription resulting in an aged phenotype. Accordingly, if expression of essential proteins is reduced or inhibited through alterations in genetic materials, a cell may lose function and/or viability, and this could be a primary cause of aging. Studies conducted over the past 50 years support these hypotheses by showing age-related alterations in DNA damage, mutation and metabolism (56,57). In addition, many of these alterations are accelerated in Werner's syndrome, Hutchinson Gilford Progeria and Cockayne syndromes, genetic diseases that show many clinical symptoms of premature aging and involve mutations in DNA repair genes. Interestingly, CR has been shown to reverse the age-related alterations in DNA damage/repair and mutations. In this article, we review the proposed mechanisms of DNA damage and repair while providing insight into current research that may assist in further understanding the molecular and cellular events involved in aging and the life-prolonging effect of CR. However, there are a variety of mechanisms by which genomic integrity can be affected. For example, the level of DNA damage and/or mutations can be affected by changes in carcinogen activation (55), increased detoxification of carcinogens, increased ability to repair DNA or a combination of these factors. CR is a well-established model for preventing the onset and delaying the progression of spontaneous and chemically induced tumors. It is inviting to suggest that an upregulation in DNA repair capacity provides a mechanistic explanation for the improved genomic stability observed in CR animals. We suggest that CR is an 'intervention' that alters the activation of specific 'stress response genes', key enzymes in DNA repair pathways, which then results in upregulation of 'DNA repair' capacity. Enhanced DNA repair reduces the levels of DNA damage, consequently

reducing mutation frequency, which would result in maintenance of genomic stability.

Mechanisms of DNA repair

The ultimate effect of any DNA alteration on cell function is dependent upon the speed and fidelity with which the damage is repaired. In replicating cells, the lasting effect of a particular damage will depend upon whether the damage is repaired before replication of the damaged area occurs. During the life span of any organism, the genome is constantly exposed to a variety of agents (58). The various insults to genomic DNA can be classified as endogenous and exogenous damages. 'Endogenous damages' include mainly replication errors, alkylating damage, and oxidative and hydrolytic damage arising from the surrounding water and cellular metabolism. Some of the hydrolytic damages encountered by the DNA are, (i) depurination/depyrimidination of nucleotides and (ii) deamination of nitrogenous bases (e.g. cytosine deamination to uracil) (59). Oxidative damage to DNA includes (i) ring-saturated pyrimidines like thymine glycols and cytosine hydrates, (ii) ring-opened purines forming FaPy products (formamidopyrimidine), (iii) 8-hydroxydeoxyguanosine and (iv) damage arising from lipid peroxidation products like M₁G and etheno adduct (59). The various 'exogenous damages' to DNA are introduced by (i) physical agents like ionizing radiations (e.g. oxidative damage), ultraviolet rays (e.g. cyclobutane dimers), and heat, and (ii) chemical agents (59). These chemical agents can be direct acting, such as alkylating agents, or indirect acting, requiring activation, e.g. benzopyrene (59).

Despite the large amount of DNA damage that occurs every day because of body temperature, metabolism and environmental insults, only a few stable changes, i.e. mutations, accumulate in the DNA sequence; the rest of the damage is eliminated with remarkable efficiency by elaborate DNA repair systems that have evolved in cells/organisms to remove all types of genomic instability (58). In mammalian cells, there is a variety of repair mechanisms—namely, direct removal of lesions, excision repair, mismatch repair and non-homologous end joining, which are each catalyzed by a different set of enzymes (60–63). Reversal of damage is the simplest repair mechanism involving a single enzyme that catalyzes the direct reversal of the damage, e.g. reversal of alkylating damage by O⁶-methylguanine DNA methyltransferase (MGMT). This repair pathway repairs both single- and double-strand DNA and requires no knowledge of the genetic information (59). Excision repair is the mechanism responsible for the repair of most DNA damage. In this repair pathway, damaged or inappropriate bases are excised from DNA and replaced by the correct nucleotide. Base excision repair pathway (BER) is believed to repair small, non-helix-distorting lesions in the DNA. The BER pathway has been estimated to be responsible for the repair of as many as one million nucleotides per cell per day (64), stressing its importance in the maintenance of genomic stability. It also is involved in the repair of the endogenous damage that arises

spontaneously in the living cell. This pathway repairs nucleotides with small base adducts like oxidized bases (e.g. 8-oxoG), hydrolytically deaminated bases (e.g. cytosine to uracil), and alkylated bases (e.g. 3-methyl adenine) (65). BER usually replaces a single-damaged nucleotide and sometimes can replace up to 6 nt, hence is classified as short-patch and long-patch BER (59,66–68). Nucleotide excision repair (NER) pathway repairs large and bulky helix distorting adducts to DNA like the ones created by carcinogens (e.g. benzopyrene, cis-platin), UV (e.g. cyclobutane pyrimidines dimers) and some oxidative adducts. NER in human cells requires about 25 proteins, including 7 associated with the genetic disease Xeroderma Pigmentosum (XP) (69). The enzymes involved in BER have strict substrate specificity, whereas the enzymes in NER have a very broad specificity, accommodating a large number of substrates. Mismatch repair pathway (MMR) predominantly repairs replication errors and also when there is insertion or deletion of nucleotides in DNA (63). In addition to the repair of damaged nucleotides and/or single-strand breaks, double-strand breaks (DSB) created by DNA damaging agents can be repaired by a specialized pathway like non-homologous end joining (NHEJ), homologous recombination, synthesis-dependent strand annealing, break-initiated repair, and single strand annealing (59).

Effect of age on DNA repair

Research over the past decades suggest that many steps in DNA metabolism are altered with age in a variety of tissues and animal models (56,57). The relation of DNA repair to aging has been studied by measuring the ability of cells from organisms of various life spans to repair DNA damage and by experiments that have compared the ability of cells from young and old organisms to repair DNA damage. Interest was peaked by the original study conducted by Hart and Setlow (70) who demonstrated a positive correlation between UV-induced DNA repair by fibroblasts with species' life span because it appeared that increased DNA repair was associated with a longer life span. A similar correlation has been shown with lymphocytes (71). However, not all studies in this area have shown an analogous relation between DNA repair capacity and species' life span (72). These conflicting results could be accounted for by the relatively crude assays often used in measuring DNA repair and the many pathways accessible to a cell for repair of damaged DNA. With the utilization of specially designed systems for evaluating DNA repair, more recent studies tend to suggest that particular types of DNA damage are not restored as efficiently in cells from old organisms.

With the advent of transgenic and knockout models, it has become possible to study complex processes by genetically manipulating individual components of the pathway or process (73). Theoretically, DNA repair pathways could be altered in two ways: repair could be reduced or enhanced. In order to enhance a specific DNA repair pathway, a systematic investigation of each component of the DNA repair pathway is necessary to determine the rate-limiting component of these

repair pathways. However, this approach has been proven difficult because components of most DNA repair pathways need to work in concert to enhance the repair capacity. Currently, the simplest approach of altering DNA repair in animal models is to utilize 'knockout' animal models. These models would potentially display a repair-deficient phenotype by reducing the activity of one essential component of the pathway. In such a transgenic model, the deficient gene product would become limiting and DNA repair reduced; thus, such animal models could potentially be used to accelerate the accumulation of DNA damage/mutations and to more directly test the Somatic Mutation Theory of Aging, i.e. these animals should exhibit accelerated aging (i.e. reduced life span), earlier incidence of disease process(es), increased changes in biomarkers of aging, and reduced lifespan. Despite difficulties, many mammalian animal models have been produced that are deficient in DNA repair genes, e.g. DNA polymerase β , XPA, XPC genes (73,74), and the impact of aging on these models has been examined. For example, DNA polymerase β heterozygous knockout mice displaying haploinsufficiency in the BER pathway, show accelerated mortality and tumor development (75). In addition, XPA homozygous knockout mice with functional NER deficiency display accelerated mutations in liver (76) and increased hepatocellular adenomas with age (77), while, homozygous XPC knockout animals show accelerated spontaneous mutagenesis with age in lymphocytes (78) and increased susceptibility to skin, liver and lung cancer in response to carcinogens (79). Interestingly, neither XPA nor XPC knockout models display accelerated aging. However, patients with trichothiodystrophy, a heritable disorder arising from point mutations in the XPD gene creating functional NER deficiency, show accelerated aging (80), while *Xpd^{m/m}* mice exhibit prominent premature aging features and a significant reduction (20%) in life span (81).

Effect of caloric restriction on DNA repair/damage

Over the past three decades, a number of investigators have compared the ability of cells to repair a variety of types of DNA damage in CR rodents. Originally, these studies were designed to determine the impact of CR on DNA repair capacity as measured by unscheduled DNA synthesis (UDS). This reputable assay measures the integration of radioactive thymidine into DNA by either autoradiography or scintillation counting after exposure of cells to a DNA damaging agent in an environment where replication is inhibited or minimal. Findings from Licastro *et al.* (82) and Weraarchakul *et al.* (53) showed that irrespective of age, dietary restriction significantly increased UDS levels after UV-irradiation in lymphocytes obtained from spleens of mice, as well as in the hepatocytes and kidney cells obtained from rats. Furthermore, using the same technique, Srivastava and Busbee (83) and Tilley *et al.* (84) measured UDS levels in hepatocytes and lung fibroblasts obtained from CR rats, supporting the view that CR enhances the ability of cells to repair the UV-induced DNA damage. In addition to UV-irradiation, Lipman *et al.* (85) observed

higher levels of UDS induced by methylmethanesulfonate in cultured skin fibroblasts obtained from CR rats as compared to animals fed *ad libitum*. Moreover, Asakura *et al.* (86) showed that cultured hepatocytes obtained from young rats treated with hepatocarcinogens, i.e. dimethylnitrosamine (DMN), on a short-term food restriction modulated the response of UDS. In contrast to the above findings, Shaddock *et al.* (87) did not observe any beneficial effect of CR on DNA repair. Contradictions in findings by Shaddock's laboratory could be due to the nature of carcinogens used by their lab. While carcinogens used by Shaddock's group required metabolic activation, other investigators used direct-acting mutagens. Inconsistencies also could arise from the use of relatively crude assays in measuring DNA repair, i.e. while UDS in combination with UV is a rather specific measure of the removal of 6-4 photoproducts by NER, UDS itself neither directly nor selectively measures the removal of a particular type of damage.

Over the last decade, research on the impact of CR on DNA repair has focused on specific DNA repair pathways and the expression of key enzymes within these pathways (Table 1). As such, understanding the mechanism of DNA repair, as well as the function of key enzymes both dependently and independently of these repair pathways has become the key to dissecting the possible beneficial effects of CR on these pathways. Originally, Hanawalt's laboratory reported heterogeneity in the repair of the dihydrofolate reductase (DHFR) gene, its upstream DNA fragments and the whole genome in response to UV-induced DNA damage (88,89). Subsequently, studies

showed that the removal of cyclobutane pyrimidine dimers (CPDs) from the transcriptionally active genes/DNA fragments was more efficient than the removal of CPDs from the non-transcribed DNA in variety of organisms, ranging from *E. coli* (90) and yeast (91) to cells from rodents (89,92,93) and humans (94,95). Thus, it was established that NER preferentially repairs transcriptionally active genes, and the transcribed strand of the transcriptionally active gene is restored more efficiently than the non-transcribed strands (88). Utilizing Hanawalt's methodology, Guo *et al.* (92) characterized how aging affects the ability of cultured hepatocytes obtained from rats to repair UV-induced DNA fragments/genes (32,92). In line with Bohr's findings, Guo *et al.* (32,92) demonstrated heterogeneity in the removal of CPDs from various DNA fragments studied. The transcriptionally active albumin gene was shown to be repaired more efficiently than the transcriptionally inactive embryonic myosin heavy chain (MHC^{emb}) gene, *H-ras* fragments and the genome overall in cultured hepatocytes (32,92). Interestingly, an age-related decline in the removal of CPD's in unexpressed DNA (i.e. MHC^{emb}), *H-ras* fragments and the genome overall was observed 24 h post-UV treatment, which was reversed by CR (32). While, Guo *et al.* (32) did not observe an age-related decline in the repair of albumin fragment 24 h post UV-treatment, an age-related decline in the rate of repair of the transcriptionally active albumin fragment was observed 12 h post UV-treatment. Intriguingly, the age-related decline in repair of albumin fragment at 12 h post UV-treatment was also reversed by CR (32).

Table 1. Effect of caloric restriction on DNA repair pathways

DNA repair pathway	Species tissue/cells	CR Diet (% of <i>ad libitum</i>)	DNA-damaging agent	Ages studied (in months)	Changes with CR	Reference
Base excision repair	Brain, liver, spleen, testes (Male Fischer 344 rats)	60		24	CR reverses age-related decline in BER by up-regulating β -pol	Cabelof <i>et al.</i> (33)
	Brain, liver, spleen, testes (Male Fischer 344 rats)	60	DMS (50 mg/kg body weight)	6	CR up-regulates β -pol and BER when exposed to oxidizing agents	Cabelof <i>et al.</i> (33)
	Brain, liver, heart, kidney (C57BL/6 mice)	60	2-NP (50 mg/kg body weight) NA	12	CR enhances uracil-initiated BER in a tissue-specific manner	Stuart <i>et al.</i> (34)
Nucleotide excision repair	Hepatocytes (Male Fischer 344 rats)	60	UV (5–30 J/m ²)	6, 12 and 24	CR reverses the age-related decline in repair of transcriptionally active strand	Guo <i>et al.</i> (32)
Mismatch repair	Small and Large Intestine (Mlh \pm C57BL/6 mice)	70	NA	Entire life span	NS difference in adenoma number but small increase in life-span seen with CR	Tsao <i>et al.</i> (99)
NHEJ ^a repair	Kidney, lung, testes, liver	60	NA	6, 12, 18 and 24	CR reverses age-related decline in ku protein in a tissue-specific manner	Um <i>et al.</i> (46)

UV = ultraviolet irradiation; DMS = dimethyl sulfate, 2-NP = 2-nitropropane, NA = not applicable and NS = not significant.

^aNon homologous end joining.

Thus, CR appears to alter the age-related decline in the rate of repair of specific genes. Also, the effect of age and CR on the DNA repair capacity of cells by the NER pathway appears to be heterogeneous in nature, i.e. the effect of age and CR is gene- as well as strand-dependent.

In addition to the aforementioned studies on the NER pathway, CR was shown to reverse the age-related decrease in DNA polymerase α -specific activity and fidelity (96). Furthermore, Prapura and Rao (97) discovered that long-term CR enhances the activity of total DNA polymerases in various regions of the brain in rats. Thus, CR appears to impact fidelity of both the DNA replication machinery as well as the component of the DNA repair pathways in rodents. More recently, Cabelof *et al.* (33) showed that CR reverses the age-related decline in BER. They demonstrated that DNA polymerase β message, protein and activity are up-regulated by CR, indicating that DNA polymerase β , the rate-limiting enzyme in the BER pathway and a stress response gene, is the specific polymerase altered in response to CR. They also showed that CR resulted in a significant up-regulation in BER and expression of DNA polymerase β in young animals as well providing them with resistance against two carcinogens: Dimethyl sulfate and 2-nitropropane. Their observations on the effect of CR on BER during aging provide an explanation for the anti-aging action of CR, supporting the hormesis theory of aging and CR.

In addition, Stuart *et al.* (34) provided evidence showing the differential effect of CR on mitochondrial and nuclear DNA-initiated BER. They observed 30% lower mitochondrial DNA damage (8-hydroxydeoxyguanosine), accompanied by a decrease in the uracil-initiated BER capacity in mitochondria isolated from brain and kidney. However, they showed a 46% decrease in mitochondrial DNA damage (8-hydroxydeoxyguanosine) accompanied by an increase in the uracil-initiated BER capacity of liver mitochondria. The reduced mitochondrial BER observed in brain and kidney could be attributed to the reduced AP endonuclease and polymerase γ activity observed during CR. They concluded that the mitochondrial genomic stability enhanced by CR was due to decreased ROS production by mitochondria rather than changes in the repair of mitochondrial DNA damage. However, Bohr's laboratory observed different effects of CR on nuclear BER (34). Uracil-initiated BER capacity in nuclei from kidney or liver was upregulated by CR. They did not observe any significant differences in the activities of DNA glycosylases (OGG1, UDG, NTH1) and AP endonuclease, supporting the observation of Cabelof *et al.* (33) that DNA polymerase β is the polymerase enhanced during CR.

Um *et al.* (46) investigated the tissue-specific changes of the non-homologous end joining (NHEJ) repair protein, Ku and the mitochondrial HSP70 in aging rats and the effects of CR on these proteins. They showed that CR reverses the age-related increase in mtHSP70 (age-related apoptotic sensitive marker) and the age-related decrease in Ku in a tissue-specific manner. Ku80/70, the regulatory component of the DNA double-strand break recognition

protein DNA-PK, is altered during aging and CR prevents this alteration. CR reverses the age-related decline in Ku80/70 in the kidney and lung with no significant difference seen in liver between old *ad libitum*-fed animals and CR animals. CR restored the Ku expression in the testis of the 18-month-old rats but was unable to restore the Ku expression in the testis of the 24-month-old rats. Moreover, Sir2, which has been suggested to play a central role in CR-induced longevity in lower organisms, (e.g. yeast), has been shown to repair DNA double-strand breaks by NHEJ, along with other proteins responsible for repairing breaks created from the recombinational repair pathway (98).

Tsao *et al.* (99) studied Mlh1 deficient mice (MMR) prone to lymphomas, intestinal adenomas and carcinomas on different diets, e.g. high, fat low calcium diet, (HFLC) and a CR diet. They observed no significant difference in the incidence of adenocarcinoma and lymphoma with CR; however, the number of adenomas was significantly greater in the HFLC-fed mice. They suggest that diet may have an enhancing effect on tumorigenesis before the onset of genomic instability caused by MMR deficiency.

Above and beyond DNA repair pathways, the levels of DNA mutation and DNA damage in tissues of rodents fed either *ad libitum* or CR diets have been ascertained by several investigators (Table 2). DNA mutations are indicative of damaged DNA that has escaped repair processes, and analysis of the frequency of mutations in the hypoxanthine phosphoribosyl transferase (*hprt*) gene is a well-established assay to quantify DNA mutation *in vivo*. Dempsey *et al.* (100) and Casciano *et al.* (101) observed significant reduction in mutations in the *hprt* gene in lymphocytes obtained from CR-fed mice and rats as compared to *ad libitum* fed animals. In addition to analysis of mutation frequency, the accumulation of DNA damage in various tissues of laboratory rodents has been shown to be reduced by CR by several laboratories. Yu's laboratory demonstrated that CR lowered the level 8-hydroxydeoxyguanosine levels (the major type of endogenous oxidative damage) in liver DNA of rats (102), and Djuric *et al.* (103) showed a significant reduction in the level of 5-hydroxymethyluracil (another oxidative product) in DNA from liver and mammary gland of rats fed a CR diet. Sohal *et al.* (104) also observed reduced levels of 8-hydroxydeoxyguanosine with CR in all tissues studied in mice, e.g. skeletal, muscle, brain, heart, liver and kidney. Kaneko *et al.* (105) also found that CR reduced the onset of the age-related increase in 8-hydroxydeoxyguanosine levels. Moreover, CR has been shown to decrease DNA damage products excreted in the urine of human subjects (106,107). In contrast to above findings, Fu *et al.* (108) showed no significant effect of CR on the accumulation of single-strand breaks in DNA in a variety of tissues obtained from mice fed a CR diet. In their analysis, Fu *et al.* (108) fed their CR mice 53% of the calories consumed by their *ad libitum*-fed controls, and the level of DNA damage was determined against control mice that consumed 80% of the calories consumed by the mice fed *ad libitum*. In their study, the control animals were not *ad libitum* fed; thus, the effect

Table 2. Effect of caloric restriction on DNA damage

DNA damage	Species tissue/cells	CR diet (% of <i>ad libitum</i>)	Ages studied (in months)	Changes with CR (% reduction)	Reference	
8-hydroxydeoxy-guanine	Brain (Male C57BL/6N mice)	60	15	34	Sohal <i>et al.</i> (104)	
	Heart (Male C57BL/6N mice)	60	15	12	Sohal <i>et al.</i> (104)	
	Kidney (Male C57BL/6N mice)	60	15	NS	Sohal <i>et al.</i> (104)	
	Liver (Male C57BL/6N mice)	60	9–17	NS	Sohal <i>et al.</i> (104)	
	Skeletal muscle (Male C57BL/6N mice)	60	23 15	35 20	Sohal <i>et al.</i> (104)	
	Brain (Male B6D2F1 mice)	60	24–26	~22	Hamilton <i>et al.</i> (111)	
	Heart (Male B6D2F1 mice)	60	24–26	~12	Hamilton <i>et al.</i> (111)	
	Liver (Nuclear) (Male B6D2F1 mice)	60	24–26	~19	Hamilton <i>et al.</i> (111)	
	Liver (Mitochondrial) (Male B6D2F1 mice)	60	24–26	~42	Hamilton <i>et al.</i> (111)	
	Kidney (Male B6D2F1 mice)	60	24–26	~12	Hamilton <i>et al.</i> (111)	
	Brain (Male Fischer 344 rats)	NA	6–24	NS	Kaneko <i>et al.</i> (105)	
	Heart (Male Fischer 344 rats)	60 NA	30 24–26 6–24	30 ~32 NS	Hamilton <i>et al.</i> (111) Kaneko <i>et al.</i> (105)	
	Kidney (Male Fischer 44 rats)	60 NA	30 24–26 6–24	34 ~50 NS	Hamilton <i>et al.</i> (111) Kaneko <i>et al.</i> (105)	
	Liver (Male Fischer 344 rats)	60 NA	30 24–26 22–24 6–24	34 ~50 NS NS	Hamilton <i>et al.</i> (111) Ward <i>et al.</i> (112) Kaneko <i>et al.</i> (105)	
	Liver (Nuclei) (Male Fischer 344 rats)	60	30 3	31 30	Chung <i>et al.</i> (102)	
	Liver (Mitochondrial) (Male Fischer 344 rats)	60 60 60	24 24–26 22–24 24	30 NS ~30 20	Hamilton <i>et al.</i> (111) Ward <i>et al.</i> (112) Chung <i>et al.</i> (102)	
	Muscle (Male Fischer 344 rats)	60 60	24–26 24–26	~50 ~55	Hamilton <i>et al.</i> (111) Hamilton <i>et al.</i> (111)	
	Brain (Sprague-Dawley rats)	60	24	~40	Wolf <i>et al.</i> (109)	
	Heart (Sprague-Dawley rats)	60	24	~50	Wolf <i>et al.</i> (109)	
	Liver (Sprague-Dawley rats)	60	24	~40	Wolf <i>et al.</i> (109)	
	Skeletal muscle (Sprague-Dawley rats)	60	24	~30	Wolf <i>et al.</i> (109)	
	Intestine (Sprague-Dawley rats)	60	24	~30	Wolf <i>et al.</i> (109)	
	Lymphocytes (Sprague-Dawley rats)	60	24	~55	Wolf <i>et al.</i> (109)	
	Liver (Mitochondrial) (Male Wistar rats)	60	24	~46	Lopez-Torres <i>et al.</i> (115)	
	5-hydroxymethyl uracil	Liver (Female Fischer 344 rats)	60	2	43	Djuric <i>et al.</i> (103)
		Mammary gland (Female Fischer 344 rats)	60	2	38	Djuric <i>et al.</i> (103)
Fpg and Endo III sensitive oxidized bases	Aorta (Male B6D2F1 mice)	60	26	~26	Guo <i>et al.</i> (29)	

(continued)

Table 2. Continued

DNA damage	Species tissue/cells	CR diet (% of <i>ad libitum</i>)	Ages studied (in months)	Changes with CR (% reduction)	Reference
Single-strand breaks	Brain (Male C57BL/6N mice)	53	6-25	NS	Fu <i>et al.</i> (108)
	Kidney (Male C57BL/6N mice)	60	6-25	NS	Fu <i>et al.</i> (108)
	Liver (Female C57BL/6N mice)	53	6-25	NS	Fu <i>et al.</i> (108)
mtDNA deletions	Brain (Male Fischer 344 rats)	60	6-24	NS	Kang <i>et al.</i> (114)
	Liver (Mitochondrial) (Male Fischer 344 rats)	60	6	NS	Kang <i>et al.</i> (114)
			18	73	
			24	71	
Mutations (hypoxanthine Phosphoribosyl Transferase locus)	Splenic lymphocytes (Female HALH/0)	60	6	NS	Dempsey <i>et al.</i> (100)
			24	20	
			24	20	

*NA = not applicable, NS = not significant and mt = mitochondrial.

of 20% restriction of calorie intake in 'control' animals may have masked the beneficial impact of CR.

More recently, Guo *et al.* (29) demonstrated that CR decreased an age-related increase in oxidative DNA damage as seen through EndoIII- and Fpg-sensitive sites in mouse aortic cells. Likewise, a significant reduction in the age-related increase in oxidative DNA damage was seen by Wolf *et al.* (109) in the brain, skeletal muscle, heart, liver, tennum mucosa and lymphocytes from rats. One major problem often ignored in measuring oxidative damage to DNA is the potential of generating oxidative damage during the isolation of DNA. Our group showed that 8-hydroxydeoxyguanosine generation during DNA isolation was eliminated using the sodium iodide isolation method (110). Using this method of DNA isolation, we measured the levels of 8-hydroxydeoxyguanosine in nuclear DNA from a large number tissues from both mice and rats (111). A significant increase in oxo8dG levels in nDNA was observed with age in all tissues studied and all strains of rodents. Thus, an age-related increase in DNA oxidation (i.e. 8-hydroxydeoxyguanosine levels) appears to be a universal phenomenon, at least in rodents. CR significantly reduced the age-related accumulation of 8-hydroxydeoxyguanosine levels in nDNA of all tissues of mice and all tissues of rats, except liver and kidney (111). In a subsequent study, our group showed that CR did indeed reduce the 8-hydroxydeoxyguanosine levels in livers and kidneys of rats (112). Thus, these data are consistent with the view that CR reduces oxidative damage in DNA in most, if not all tissues of rodents.

In addition to the protection conferred by long-term CR on nuclear DNA from various endogenous and exogenous factors and oxidative damage, CR has also been shown to protect mitochondrial DNA from oxidative damage. A 20–25% reduction in the level of 8-hydroxydeoxyguanosine in rat liver mitochondrial DNA has been shown by CR (103). Using the sodium iodide isolation method, we observed an age-related

increase in 8-hydroxydeoxyguanosine levels in mtDNA isolated from the livers of rats and mice, and this increase was significantly reduced by CR (111). Moreover, Melov *et al.* (113) showed that CR significantly reduced the age-related accumulation of mitochondrial DNA mutations in heart tissue of mice. More recently, Kang *et al.* (114) witnessed a reduction in age-related mitochondrial DNA deletions in the rat liver by CR. In contrast, the brain of these animals exhibited no alteration in the level of mitochondrial DNA deletions by CR. Monica Lopez-Torres *et al.* (115) showed that CR for 12 months reduced oxidative damage to mitochondrial DNA by 46%. Short-term CR (6 months) reduced mitochondrial ROS formation by 23%, and reduced significantly 8-hydroxydeoxyguanosine levels in mitochondrial DNA. However, no difference was observed in the oxidative damage in nuclear DNA compared to the old *ad libitum* control animals. They suggested that the difference in oxidative damage to nuclear DNA and mitochondrial DNA could be due to the efficient repair of nuclear DNA and that the reduced 8-hydroxydeoxyguanosine by CR in mitochondrial DNA was due to the reduced ROS production.

Overview and future directions

The concept of the 'anti-aging' and 'anti-cancer' actions of CR has come a long way since its discovery some 70 years ago. It is now clear that the increase in the survival of rodents by CR is due to the restriction of calories (14). Studies over the last three decades have refuted some of the previously proposed mechanisms, e.g. alteration in growth, body weight and metabolism, as the sites of CR action. Currently, the major hypotheses advanced to explain the mechanistic basis for the protective effects of CR are: oxidative damage attenuation, altered glucose–insulin system, and hormesis (14). Perhaps the strongest evidence in support of the above proposed mechanisms is data from studies in which aging has been retarded by CR or studies of genetically accelerated aging. Based on

the data collected over the last few decades, it is becoming clearer that perhaps the anti-aging action of CR is due to the collective actions of different mechanisms, impacting the capacity to adapt to environmental stimuli by enhancing repair/maintenance status such as antioxidant defenses and DNA repair capacity. In line with this view, the hormesis hypothesis has become an attractive proposal explaining CR's ability to 'increase longevity, retard senescent deterioration, retard age-associated diseases, and enhance coping with environmental stimuli' (14). In other words, the hormesis hypothesis embraces many of other proposed mechanisms, suggesting that while high intensity stress may trigger pathology and death, low intensities or concentrations of environmental challenges would lead to a beneficial change. Thus, under hormesis, DNA repair capacity would become a relevant mechanism where CR is capable of enhancing stress response genes such as DNA polymerase β , leading to resistance to stress and enhanced longevity (14,33,116).

As outlined in this review in support of hormesis hypothesis, many laboratories have shown the role of DNA repair capacity as a beneficial action of CR through increased DNA repair in cells from rodents (Table 1), reduced levels of oxidative damage in DNA, and reduced levels of mutations (Table 2). Therefore, under hormesis, CR attenuates the rate of accumulation of damage by enhancing DNA repair response through upregulation in the expression of stress response genes, thereby retarding senescent deterioration and perhaps extending life span. However, if CR's ability to extend life span is due to hormesis, will CR enhance the stress response even in young mice where no aging phenotypes are observed? Interestingly, in an extensive study, Cabelof et al. (33) examined the effect of CR on the BER pathway in young mice and rats. While CR was shown to reverse the age-related decline in BER, as well as DNA polymerase β activity, protein, and mRNA levels (33), CR also reversed mutation frequency in young animals exposed to DNA damaging agents. In support of Cabelof's finding and to address whether CR is beneficial earlier in maturation, Shima *et al.* (117) measured the effects of CR during murine development, and discovered that during development, CR is moderately protective against chemically induced mutagenesis. Thus, perhaps the low-intensity environmental challenge exerted by CR even in young mice resulted in beneficial effects via enhancement of the stress response genes in DNA repair pathways. It becomes attractive to suggest that CR may confer protection from endogenous DNA damage at a young age; i.e. at a very early stage of initiation, CR can prevent fixation of DNA damage, which might then delay and/or prevent initiation of cancer and delay aging. Consistent with this suggestion is the observation that a decrease in spontaneous mutation frequency is observed in 6-month-old CR mice (33). Intriguingly, the capacity of short-term CR to provoke phenotypic changes noted by long-term CR (118) suggests that genomic imprinting occurs early with the inception of CR and that the early alterations in expression of key DNA repair genes observed are a means to the life-extending capability of

CR. With respect to DNA repair, and excision repair in particular, the possibility that CR has immediate and long-term effects on the protection of genomic integrity is compelling. It suggests that CR is not acting solely through reversal of age-accumulated changes, but that it has specific and direct effects on expression and activity of repair/maintenance pathways, e.g. the BER pathway, which would protect organism from endogenous and environmental damages as well as support life-long maintenance of genomic integrity. Thus, the use of animals with targeted disruptions in specific DNA repair pathways, e.g., BER genes, will allow further analysis of the role that DNA repair may play in the anti-tumorigenic and anti-aging actions of CR. Further, it will be possible using transgenic models to determine whether CR prolongs lifespan through alterations in the DNA repair machinery of the animals. The data from such experiments will provide investigators with first-hand knowledge of the mechanism by which CR reduces DNA damage/mutations and prolongs life span. In summary, the recent studies on the effects of CR on DNA repair/damage are quite interesting and most results are in favor of the view that changes in DNA repair/damage play an important role in aging. However, additional experiments that employ more accurate assays of DNA repair are required to establish definitively that CR alters DNA repair/damage.

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