

Oxidative Stress and Calorie Restriction in Aging

Byung Pal Yu^{1†}, Byung Ou Lim² and Hae Young Chung³

¹Department of Physiology, University of Texas Health Science Center, San Antonio, TX

²Department of Applied Biochemistry, Konkuk University, Chungju

³Longevity Life Science & Technology Institutes, Department of Pharmacy, College of Pharmacy,
Busan National University, Busan

Introduction

Oxidative stress refers to a condition whereby imbalances in the redox state causes oxidatively modified cellular constituents and dysfunctions. Data from various sources overwhelmingly made clear that overall oxidative stress is elevated during aging (Warner and Starke-Reed, 1997; Beckman and Ames, 1998). For those who are interested in oxidative stress and calorie restriction (CR) in aging, and wants to keep abreast of these rapidly expanding fields, a new bioinformatic database should be consulted (Park *et al.*, 2004). Among the most relevant and impressive mechanisms underlying calorie restriction (CR) in retarding the aging process are its abilities to resist age-related oxidative stress and to maintain a proper redox balance. It has been said that if CR has no ability to modulate age-related oxidative stress as caustic factors in the aging process, CR would lose its strong supportive basis (Yu, 1994)

The original free radical theory of aging was proposed in 1956 (Harman, 1956). The basic tenet of this theory is that superoxide and hydrogen peroxide, generated from the aerobic metabolism of organisms, are intrinsic factors capable of destroying both cellular structure and function, which lead to the pathogenesis of many diseases that leads to aging. However, information generated during the last several decades necessitates the modification of the original free radical theory, because when the original theory was proposed, our knowledge of both aging and free radicals were in their infancy. Therefore, understandably, what we know now as most the essential aspects of free radical biology

and aging were not addressed in the original form. Thus, it is prudent to know why the oxidative stress hypothesis was proposed (Yu and Yang, 1996; Yu, 1996), as briefly described below.

Distinctions between free radical theory and oxidative stress hypothesis of aging.

Current knowledge gained from both the fields of aging and free radical biochemistry has revealed the shortfalls in the original free radical theory (Yu, 1996). Three major aspects of the free radical theory need amendments: 1) inclusion of various reactive species, such as singlet oxygen, nitrogen-derived NO, its derivatives, and reactive lipid products, such as lipid hydroperoxides and aldehydic species, which were not considered in the original free radical theory; 2) essentiality of defense systems and the maintenance of the redox balance; and 3) consideration of biological aging as primary process and age-related disease as secondary process, not the reverse as was in the original version.

To fulfill these deficiencies, an oxidative stress theory of aging was proposed (Yu and Yang, 1996). This theory is particularly meaningful when one considers possible interventions and anti-oxidant strategies. For instance, because of the multiple types of reactive species, no single anti-oxidant is capable of fending off the large a variety of reactive species; the most effective anti-oxidative measures require multi-functional efficacies and the ability to neutralize a broad spectrum of reactive species. The defense systems emphasized in the oxidative stress theory are essential biological components for the proper maintenance of redox balance, as important as the delicate intracellular pH balance in regulating homeostatic mechanisms such as signal transduction activity (Kim *et al.*, 2002b).

[†]Corresponding author

Tel: 1-760-451-2045 Fax: 1-425-675-3578

E-mail: bpyu@earthlink.net

The last point of oxidative stress theory making an important distinction is its stance that biological aging and pathological processes have separate underlying mechanisms. This distinction signifies that aging is not a disease; as we well know, the aging process occurs in the absence of disease. Thus, according to this view, therapeutic treatments on any specific disease would have only limited success as an anti-aging intervention. It is important to recognize that CR is currently the only experimental paradigm shown clearly to modulate both processes (Merry, 2002).

How CR exhibits its anti-oxidative action against oxidative stress

Accumulated evidence has proven CR to be the most effective modulator of oxidative stress (Sohal and Weindruch, 1996; Yu, 1996). The oxidative stress hypothesis offers explanations on how CR can build up defenses and resistance against oxidative stress. Investigations looking into the oxidatively stressed animals, consistently found a disrupted redox status, which was conversely well preserved in CR animals. Studies show that free radical generation (Sohal and Weindruch, 1996) and lipid peroxidation (Yu, 1996) are suppressed by CR, while boosting the defense systems (Cho *et al.*, 2003). Weakened defenses and redox imbalance, i.e. oxidative stress, cause alterations in cellular structural constituents, such as DNA, membrane lipids, and proteins, but in caloric-restricted organisms, all the oxidative alternations are shown to be blunted (Yu *et al.*, 1999; Lopez-Torres *et al.*, 2002). The biochemical modifications of cellular constituents, like collagen by oxidative-induced glycation during aging, is shown to be suppressed by CR in a nonhuman primate study, offering another good illustration of CR's anti-aging effects (Sell *et al.*, 2003).

Studies show that damage to both nuclear and mitochondrial DNA and the membrane instability of mitochondria and microsomes occurs with age are all attenuated by CR (Yu, 1996). Functionally, many uncontrollably activated redox-sensitive transcription factors with age are blunted by CR (Kim *et al.*, 2002a) resulting in well-regulated cellular signal transduction pathways. Because many forms of age-related diseases, neoplasms in particular, have been linked to oxidatively modified cellular constituents and functions, the suppression of oxidative stress by CR may be the likely mechanism for its beneficial action in the prevention of many age-related disease processes (Yu, 1994; Warner

and Starke-Reed, 1997).

Protection of membrane integrity by CR

The dynamic, yet tightly structured cell membrane has been the subject of many continuing research endeavors (Vereb *et al.*, 2003). The importance of membrane deterioration in aging was highlighted in the membrane hypothesis of aging proposed (Zs-Nazy, 1994). In a recent publication, A. J. Hulbert proposed the membrane pacemaker hypothesis of aging that is based partly on CR's action in modifying the fatty acid profile of membrane bilayers, resisting membrane lipid peroxidation, and extending life span (Hulbert, 2003).

Although the membrane damage by free radicals has been the topic of many reports in the past, not much serious attention was given to the membrane lipid peroxidation or its consequences in the aged membrane. Because of the membrane's high lipid content, and a generation source of reactive species, membrane stability is strongly dependent on the integrity of the fatty acids, as unstable polyunsaturated bonds are preferential damage sites of oxidative targets (Laganieri and Yu, 1993). Evidence on age-related oxidative stress comes from life-long, longitudinal measurements of pentane levels in exhaled air, as a marker for *in vivo* lipid peroxidation, showing CR rats with much lower pentane levels than the AL rats (Matsuo *et al.*, 1993a).

Protection by reduction of lipid peroxidation

The gerontological literature shows substantial evidence that the membranes of aging organisms lose their resilience and become rigid with time due to the loss of membrane fluidity from mainly oxidatively modified fatty acids and its reactive byproducts (Chen and Yu, 1994; Choe *et al.*, 1995). This change in membrane fluidity seems to be widespread, occurring in most tissues, whether they are mitotic or postmitotic (Pieri, 1997; Pamplona *et al.*, 1998). Generally, changes in membrane fluidity was explained in two ways: 1) changes caused by increased saturation or decreased unsaturation of fatty acid composition, and 2) changes caused by increased membrane cholesterol content causing the membrane to become condensed, i.e., rigid (Choe *et al.*, 1995; Lee *et al.*, 1999). Several old data recorded in gerontological papers show such occurrences in aged membranes (Zs-Nazy, 1994).

One misconception points to increased cholesterol as the culprit in age-related membrane rigidity. However, findings from membrane lipid peroxidation studies (Yang

and Yu, 1993; Choe *et al.*, 1995) revealed that the age-related changes in membrane fluidity that cause membrane rigidity from lipid peroxidation and its peroxidized lipids, not from increased saturation or cholesterol. Evidence shows that lipid peroxidation and peroxidized lipids, when compared to cholesterol, are far better inducers of membrane rigidity (Lee *et al.*, 1999). Further, because mitochondrial membranes are major sites of reactive species production, both mitochondrial structure and its membrane-associated functions are exquisitely sensitive to lipid peroxidation (Lee *et al.*, 1999). It is interesting to note that deleterious changes in the mitochondrial membrane (e.g., rigidity) takes place as young as 6 and 12 months in *ad libitum*-fed animals, as opposed to CR animals (Pieri, 1997; Lee *et al.*, 1999). As expected from its anti-oxidative action, CR can effectively suppress age-related lipid peroxidation-induced membrane rigidity and maintain membrane integrity throughout life.

Protection by changing membrane fatty acid profile

The membrane resistance to oxidative stress is especially important to cellular homeostasis because of the essential roles in protecting the membrane permeability, membrane potential, the membrane receptor and signal transduction systems (Kristal and Yu, 1998a). CR as a powerful membrane protector exhibits a clever manipulation by rearranging the membrane fatty acid composition profiles to resist oxidative attack (Laganieri and Yu, 1993; Pamplona *et al.*, 1998). This is a surprising revelation because no such changes in fatty acid composition have been reported from simply reducing calorie intake, although many dietary lipids have been known to influence the membrane fatty acid composition (Pieri, 1997).

It was found that CR rearranges the membrane lipid composition by decreasing polyunsaturated fatty acids (PUFA) such as 22:4 and 22:5, while replacing PUFA with less peroxidizable, 18:2, or 18:3 fatty acids (Laganieri and Yu, 1993). By this manipulation, proper membrane fluidity is maintained with a reduced risk for peroxidation. This interesting manipulation is deemed as part of the organism's innate defense strategy to protect the membrane against increased peroxidizability. This survival strategy seems to be an adaptive trait gained through the evolutionary process, as shown in the work of Pamplona (Pamplona *et al.*, 1998) who found an inverse relationship between the amount of

PUFA, (i.e., increased peroxidizability) and the life spans of several animal species, including mice, rats, pigeons, horses, and long-living humans: the higher the PUFA content in the membranes, the shorter the life span—similar to findings with *ad libitum*-fed and CR rats.

Additional data support membrane fatty acid damage with age, as evidence by the increased membrane phospholipase A₂ in *ad libitum*-fed rats (probably in response to increased peroxidized lipids) and the reduced levels of this enzyme in CR rats (Yu *et al.*, 1992; Yang and Yu, 1993). Additional corroborating data comes from a study in which delta-6 desaturase, a rate-limiting enzyme in the conversion of linoleic acid (18:2) to PUFA, is significantly lower in CR rats than in *ad libitum*-fed rats. Thus, it seems that organisms with a restricted calorie intake has multiple ways to reduce age-related lipid peroxidation, i.e., by replacing PUFA with more stable, but less rigid 18:2 or 18:3 unsaturated fatty acids, and by regulating selective desaturases, as shown by delta-9 desaturase.

Protection of Mitochondria by CR

The early sign of oxidative modification occurring in the mitochondrial structure during aging is increased levels of peroxidized mitochondrial lipids. The amount of lipid hydroperoxide was observed to be much higher in mitochondria taken from old *ad libitum*-fed rats compared to the levels found in CR rats (Yu, 1994). The significance of these high levels of peroxidized lipids is that not only they can cause structural disruption, but more dangerously, peroxidized lipids generate various reactive lipid species such as aldehydic 4-hydroxynonenol (HNE) (Uchida, 2003). Evidence shows increased accumulations of degraded lipid products are more potent within the mitochondrial membrane during aging (Chen *et al.*, 1995), because they are long acting, easy to diffuse, and have no specific scavengers for them, unlike most free radical derivatives. Again, the diverse actions of CR effectively prevented damage from HNE.

Mitochondrial sensitivity to reactive aldehydes was shown in mitochondrial transcription activity (Kristal *et al.*, 1994a). Proper transcription function requires well-maintained mitochondrial gene expressions of many respiratory enzyme proteins. Although the susceptibility of these functions to oxidative stress by oxygen-derived free radicals has been suspected, the effect of lipid peroxidation has not been well examined until recently. Published data indicate that HNE perturbs the inhibition

of mitochondrial transcription activity as effectively as peroxy radicals. CR's anti-peroxidative action certainly has been shown to work well in the maintenance of transcription function by reducing the amount of reactive aldehydic compounds (Kristal *et al.*, 1994b).

Exposure of mitochondria to various oxidants causes mitochondrial deterioration with concurrent functional loss. One functional change sensitive to oxidant treatment is mitochondrial permeability transition (MPT) (Kristal and Yu, 1998a). An age-related increase in MPT of liver mitochondria isolated from male Fischer 344 rats, 6-24 months of age, was shown to be due to deteriorated membranes, which was reversed by CR (Kristal *et al.*, 1996) showing that CR regimens greatly delay the opening of the MPT Ca^{2+} mega channel upon exposure to tert-butyl hydroperoxide, thereby indicating resistance of mitochondria to oxidative stress. The increased resistance to MPT induction was maintained through 24 months of age in CR animals with the resilient nature of the membrane.

The significance of mitochondrial membrane protection and well-regulated MPT by CR could provide possible clues for the differences noted between *ad libitum*-fed and CR animals in mitochondrial proton leaks, as reported in recent interesting papers (Lal *et al.*, 2001; Lambert and Merry, 2004).

A most recent paper raised interesting questions regarding the counteraction of insulin against the inhibitory effect of CR on the mitochondrial hydrogen peroxide generation (Lambert and Merry, 2004). The authors found that CR was able to decrease the mitochondrial proton motive force that resulted from an increased proton leak activity and decreased substrate oxidation. Interestingly, a two-week treatment of insulin attenuated the CR effect. Although the exact mechanism for the insulin counteraction is unknown, the authors speculate that under CR conditions, a reduced substrate and increased proton leak (due to reduced insulin) could lead to a lower the electrochemical energy gradient and a lower production rate of reactive species (Lal *et al.*, 2001). The significance of this finding is that a possible link between oxidative stress and insulin-related metabolic processes, and should be explored further (Lambert and Merry, 2004).

Apoptosis, a programmed cell death, is a normal function of an organism's tissue development and maintenance (Warner and Starke-Reed, 1997; Higami and Shimokawa, 2000). One important point of apoptotic induction is its responsiveness to reactive species and

oxidative stress. This relatively new area of study has attracted the attention of aging researchers, who have documented some interesting data on age-related apoptosis (Ando *et al.*, 2002), although there is some debate on exactly how big a role apoptosis plays in the aging process per se.

Many consider apoptosis to play an important role as part of a defense mechanism in the protection against cell or tissue damage, and accumulated evidence indicates that age-related apoptosis may be tissue- and organ-specific as seen in increased apoptosis of mitotic tissues and cells (Higami and Shimokawa, 2000; Julian and Leeuwenburgh, 2003). Mitochondrial participation in apoptosis seems to be essential: Many mitochondria-associated factors, including cytochrome c, redox-sensitive genes, like Bcl-2, Bcl-x, and pro-apoptotic Bax, and proteolytic caspases play major roles in regulating the apoptotic activity, which seems to be influenced by age and oxidative status of the mitochondrial membrane.

The effect of CR's mitochondrial protection can be seen in age-related apoptotic activity. Rajani *et al.* reported that the incidence of apoptosis increased with age in rat brain and that CR attenuated this activity (Rajani *et al.*, 2003). However, in the liver, animals on short-term CR (two months) showed significantly higher apoptotic activity than controls. Age-related mitochondrial membrane dysfunction, which has well been documented, can influence the regulation of the apoptotic process by releasing cytochrome c or anti-apoptotic Bcl-2 from mitochondria in coordination with caspases and pro-apoptotic Bax. In the kidney, it was found that CR effectively prevents the release of cytochrome c, upregulates Bcl-2, and reduces Bax, leading to the suppression of the apoptosis process.

Age-related DNA damage and modifications have been linked to many cellular dysfunctions and age-related diseases. Rao suggested recently that one of the life-extending mechanisms of CR might stem from its ability to channel limited energy resources to maintain essential processes, like DNA repair, rather than towards reproductive and anabolic activities (Rao, 2003).

Mitochondrial DNA (mtDNA) is also subject to oxidative stress during aging. Several laboratories have already hypothesized that compared to nuclear DNA, mtDNA is probably more susceptible to oxidative stress. Similar conclusions were reached based on reports showing oxidatively modified DNA in rodent models. Fraga (Farga *et al.*, 1990) and Richter (Richter *et al.*, 1988) have shown that the levels of oxidatively damaged

mtDNA found in rat liver are about 10-fold higher than those found in nuclear DNA. Additional data on CR's ability to protect-DNA from both nuclear and mitochondria damage were reported by Chung *et al.*, who found about a 15-fold higher level of 8-OHdG in mitochondrial DNA compared to nuclear DNA, for which CR attenuated damage in both (Chung *et al.*, 1992).

Mitochondrial DNA deletions causing various genetic abnormalities are also well documented. One interesting finding is the possible causality of the abnormalities cross-links mtDNA to lipid peroxidation (Hruszkewycz and Bergtold, 1990). Data supporting their findings on mtDNA alterations are reported. For example, in aging rats, mtDNA deletions clearly showed a 6-fold increase in liver mitochondria between ages 6 and 24 months. One salient point of this finding is that the CR's anti-oxidative action effectively suppressed the age-related mtDNA deletions (Kang *et al.*, 1998).

One interesting paper recently reported (Hoffmann *et al.*, 2004) negative data, showing that reactive oxygen species derived from the mitochondrial respiratory chain are not responsible for the basal levels of oxidative modifications to nuclear DNA. Further investigation is warranted because endogenous long-acting reactive lipid species like 4-HNE, rather than short-lived, less diffusible and charged free radicals, are can cause more serious damage for a long-lasting age-associated consequences.

One word of caution is the sensitive measurement on any membrane-associated activities like proton leaks, receptor functions or cytochrome c releases, which are all influenced by how well mitochondria were prepared and whether integrity was maintained during the isolation procedure. We already know that aged mitochondria from *ad libitum*-fed animals are more fragile than young mitochondria. Damage due to improper technical procedures could lead to erroneous and exaggerated results. Such a major concern was already reported by two publications (Sastre *et al.*, 1996; Hagen *et al.*, 1997).

Modulation of gene expression by CR

With interest to gene modulation during aging, several lower organisms have been used to study age-related genomic changes during aging (Plethcher *et al.*, 2002). The consensus obtained from these studies is that aging, characterized by structural and functional alterations of physiological systems, exhibit the same degree of dynamic change in the molecular expression (Weindruch *et al.*, 2002). As a potent anti-oxidative modulator, CR exhibits remarkable effects in how genes

are regulated, particularly the redox-responsive genes. In the following sections, some of major findings involving the CR paradigm studies are described.

With the advent of the high-density oligonucleotide microarray method for a screening more than 7,000 genes, researchers were able to show that aging leads to the selective activation of transcripts known to be involved in oxidative stress and inflammation and that the suppressed genes involved in mitochondrial electron transport and oxidative phosphorylation were attenuated in CR mice (Lee *et al.*, 2002). In a more recent study, microarray analysis of 11,000 genes in the liver was reported (Cao *et al.*, 2001). It is interesting to note that many of the genomic responses to CR were adjusted quickly within a matter of a couple of months (Dhahbi *et al.*, 2004). Interestingly, modulated genes were associated with inflammation, oxidative stress, and DNA replication, similar to those data from muscle of monkeys.

Transcription factors are so named by their function in the transduction of cellular signals to the transcriptional process. They are bound to specific DNA sequences, and many of them are redox-responsive. Among many transcription factors, NF- κ B is known to be exquisitely sensitive to oxidative stress, and the chronically activated NF- κ B during aging is readily down regulated by CR (Kim *et al.*, 2002a; Kim *et al.*, 2002b). The significance of the age-related activation of NF- κ B is that it regulates unusually a large number of genes involved in many normal and pathological processes like immune and inflammatory processes (Chung *et al.*, 2000). The inflammatory process is now recognized as part of many age-related, chronic disease processes, including arthritis, vascular aging, cardiovascular diseases, cancer, and dementia (McGeer and McGeer, 1999; Colwell, 1999; Teunissen *et al.*, 2003). Molecular probing of the age-related chronic activation of NF- κ B supports this conclusion.

It was found the age-related activation of NF- κ B is enhanced by the degradation of its subunits, I κ B, allowing NF- κ B to translocate into the nucleus during aging (Kim *et al.*, 2002a). Moreover, in another recent study, it was that the CR's effective suppression of the age-related NF- κ B activation was carried out by the inhibiting the dissociation of I κ B from NF- κ B, thereby, leading to the suppression of the inflammatory process (Chung *et al.*, 2000).

The anti-inflammatory action of CR

Recent research has produced convincing data

implicating oxidative stress as a major causative factor eliciting the inflammatory process (Chung *et al.*, 2001). Although the detailed mechanism has yet to be worked out, the involvement of redox-responsive transcription factors play a major part in creating the pro-inflammatory state seen with aging. During aging, the up-regulation of pro-inflammatory cytokines, TNF α , and iNO synthase occur, and CR suppresses this age-related change (Chung *et al.*, 2001).

The suppression of inflammatory process by CR is further demonstrated by the attenuation of the age-related up-regulation of COX-2 gene expression and pro-inflammatory cytokine synthesis, like IL-6, which are shown to be suppressible under reduced oxidative stress, as in the case with CR.

Based on observations of the pro-inflammatory status in the aged, the broad involvement of inflammation in many major chronic diseases, and CR's prevention against the activation of the pro-inflammatory transcription factors, the molecular inflammation hypothesis of aging was proposed (Chung *et al.*, 2001; Chung *et al.*, 2002). This hypothesis identifies the age-related inflammatory process as a possible molecular cross-talk mechanism that bridges biological and pathological processes (Chung *et al.*, 2002). The anti-inflammatory action of CR can be attributed more likely to its ability to suppress age-related oxidative stress, than to a chronically elevated glucocorticoid level, which is potentially deleterious to the organism (Sapolsky *et al.*, 1986; Sabatino *et al.*, 1991; Patel and Finch, 2002). The report (Lee *et al.*, 2000) showing CR's selective suppression of glucocorticoid receptor expression in the hippocampus and cerebral cortex is supportive of such conclusion (more discussion in below).

Resistance to disease by CR

CR's ability to cause an organism's resistance to various stressors is perhaps best exemplified when animals were subjected to life-threatening toxic agents or radiation, as shown in the 1942 pioneering work of Tannenbaum using mouse skin tumors induced by benzo(a)pyrene (Tannenbaum and Silverstone, 1953). A report by Chu revealed further insights into how CR enhances resistance by inhibiting the interaction of DNA with the potent carcinogenic aflatoxin B (AFB) (Chu *et al.*, 1991). These investigators found that AFB-induced hepatic tumors are reduced by more than 50% in CR rats. Moreover, CR reduced AFB-DNA adduct formation by as much as 71% in these rats, depending on the

adduct type, compared to control rats. Furthermore, the authors found that *in vitro* nuclear DNA binding of AFB is 37% lower in CR rats than in controls, although exposure to activated AFB was the same for both groups. A similar resistance was shown in a DNA strand break experiment. A more stable double-stranded DNA was maintained following alkaline treatment in CR rats, whereas an approximate four-fold increase in damaged single-stranded DNA was found in *ad libitum*-fed rats.

A study (ThyagaRajan *et al.*, 1993) showed CR's ability to resist against hormone-inducible tumorigenesis. This group investigated the mechanism by which CR suppresses carcinogen-induced mammary tumors in the rat and whether CR rats have the ability to blunt the action of tumor-promoting estrogen and/or prolactin. Their results show that when challenged with these tumor-promoting hormones, CR, indicating again a strong ability to resist tumor growth even under powerful hormonal stimulation by cancer-promoting estrogen and prolactin, significantly suppressed tumor progression.

An even more remarkable example of CR's ability to resist irradiation is shown in the work of Gross and Dreyfuss, 1990, who challenged mice with gamma irradiation to induce tumorigenesis. They not only show a clear-cut tumor suppression by CR, but also that CR rats had a far stronger ability of resisting one of the most deleterious forms of stressors. While 43 out of 89 (48%) of the no irradiated control, *ad libitum*-fed rats showed tumors, none of 77 irradiated rats on the restricted diet developed tumors.

It would be interesting to know whether the suppression of the tumorigenesis by CR is related to the delay in the onset or the progression of the disease process (Shimokawa and Higami, 1994). Although there is no easy way to test this question, re-analyses of earlier works can shed some light on the CR effect against the incidence of leukemia in male Fischer-344 rats (Shimokawa *et al.*, 1993). Correlative pathological data on neoplastic lesions and the survival data led to the conclusion that CR delays only the onset of the disease without modulating the progression or the course of leukemia in late life.

Stress resistance by CR for longevity

When exposed to a certain stressor, an organism's adaptive response undergoes three interrelated stages: alarm reaction, resistance, and exhaustion, according to Selye's definition (Selye and Tuchweber, 1976). Because stress itself is a most effective factor in eliciting adaptive

responses, the organism's innate nature is to use some form of stress for its own benefit. Such a notion is in line with the basic premise of the proposed stress theory of aging and adaptation hypothesis for longevity (Parson, 1993).

Hardly an aspect of aging is more important than an organism's ability to withstand stress or to resist both internally and externally imposed insults. We know that as organisms lose their ability to resist these insults, aged organisms suffer more than the young. Therefore, the prime strategy for an organism's survival has been the evolutionarily adapted defense systems that guard against an insult, somewhat like to oxidative stress. Although terms like stress, resistance, and adaptability have long been used in biology, they remain mechanistically and quantitatively poorly defined. In a gerontological context, stress resistance or susceptibility is often discussed in association with an organism's vulnerability to disease and age-related damage (Yu and Chung, 2001).

The life-prolonging action of CR seems to offer an excellent opportunity for investigating the interrelationship between stress and the aging process. As an omnipotent intervention, CR provides a unique opportunity to probe an organism's ability to withstand age-related stress as a survival strategy. In this context, the anti-aging action of CR can be viewed as "nutritional or metabolic stress", because the organism's reduced caloric intake seems to be a stimulatory metabolic response for survivability. Recent gerontological research has provided sufficient experimental data supporting this anti-aging property of CR, of which several pertinent, key examples are discussed below

Evidence of stress resistance by CR

One well-known, exemplified response to stress is the hormonal increase in adrenal corticosterone levels in plasma during aging, where increases in these levels appear to be proportional to the degree of stress. Aged animals appear to have a diminished ability to attenuate the increase, causing the aged to have continually elevated plasma levels of corticosterones. These authors suggest that increased levels of corticosterone in aged rats result in hippocampal neuronal cell death, that is, the stage of exhaustion (Sapolsky *et al.*, 1986). However, this scenario in the glucocorticoid cascade hypothesis is obviously not applicable in the case of the CR paradigm, because CR results in an increased life span in spite of chronically elevated diurnal levels of serum corticosterone

(Sabatino *et al.*, 1991). This apparent contradiction makes the interrelation of glucocorticoid and aging far more complex than one might want to narrowly define it and needs other mechanistic explanations (Lee *et al.*, 2000; Patel and Finch, 2002).

Frame viewed elevated glucocorticoid as the major adaptive response to nutrient stress (Frame *et al.*, 2001) Data clearly show that biologically active free corticosterone levels in CR rats are higher than those in *ad libitum*-fed rats throughout the animals' life spans. Interesting and more important questions, however, are, how do chronically elevated, deleterious glucocorticoid levels cause no apparent harm to CR animals, and do how these animals use it to their own advantage. The answers can, in part, be found by CR's ability to resist corticosterone-induced neuronal membrane damage, and/or the corticosterone reduction of receptor (Lee *et al.*, 2000) as described in previous section.

Synergistic effects of CR and exercise

A consequence of the increased metabolic demand that physical exercise elicits is increased oxidative stress, as indicated by the increased production of oxidants in mitochondria, which seems paradoxical for what said about the anti-aging effects of exercise. A report shows that exercised CR rats are shown to have an additional protective defenses against oxidative stress compared to their non-exercised CR counterparts (Kim *et al.*, 1996a,b). An interesting question arises: How can this be possible if exercise promotes oxidative stress? The answer could come from the CR's unique ability to mobilize a series of adaptive defense mechanisms. For example, in addition to the enhanced anti-oxidative scavengers, the free radical generation of microsomes, in contrast to mitochondria, was shown to be significantly suppressed by exercise in CR rats, even at 20 months old (Kim *et al.*, 1996a,b).

Another interesting manipulation by CR is its ability to resist exercise-induced oxidative stress on membrane integrity (Kim *et al.*, 1996b). Data show that exercised CR rats, despite having the increased mitochondrial reactive species production, can maintain mitochondrial membrane fluidity as good as that of young sedentary rats. The mechanisms for such remarkable resistance to stress are likely to be derived from a concerted network of stress- response elements, for example, lowered membrane peroxidizability and antioxidant defenses. As a consequence, the membrane fluidity can be better preserved by exercise.

CR as a biological hormesis

Numerous studies on CR have established its efficacy as the most effective life-prolonging intervention known today, extending both average and maximum life spans. Emerging views on extended longevity lead us to believe that CR's resistive action against stress is an evolutionary, adapted measure, which is characteristic of a hermetic response (Calabrese and Baldwin, 1998).

The concept of hormesis may well offer a biological basis for the CR phenomenon (Hoffmann and Hercus, 2000; Rattan, 2004). The term *hormesis* by definition describes a beneficial, biological effect at low levels, as seen with reduced caloric intake, which at higher levels would cause deleterious effects (Neafsey, 1990). The evidence from genomic profiling data strongly suggests an organism's adaptive response to CR turns on selective genes essential that are necessary to maintain high metabolically efficient state for the survival of the organism. The maintenance of homeostasis by adapting to stress during aging is likely the key determinant for extended longevity, as observed with the CR paradigm.

Future Directions for CR Research

The important question one should ask is whether or not the anti-aging effects of CR observed in laboratory animals and nonhuman primates can be applicable to human aging; the answer could come the foreseeable future as two groups of investigators in the United States have been conducting CR studies on nonhuman primates (Mattison *et al.*, 2003) over 13 years. Their results so far are encouraging in that most of the data on monkey studies confirm the data collected on lower animals. With this experimental information, we will be one step closer to the applicability of this nutritional paradigm to humans (Walford *et al.*, 2002; Heibronne and Ravussin, 2003). It is worthy noticing of a most recent publication that indicates a significant reduction of incidence of breast cancer in Swedish women by CR (Hursting *et al.*, 2003).

Some recent developments in CR research could turn out to be significant in revealing more information on the underlying molecular mechanisms of CR. For instance, a recent report (de Cabo *et al.*, 2003) showed this group's attempt to develop an *in vitro* model of caloric restriction model by treating cultured cells with sera obtained from CR rats or monkeys. Their results showed that cultured cells treated with CR sera had reduced cell proliferation, enhanced tolerance to oxidative stress, and increased heat stress-response gene

expression, similar to those animals on CR. Another interesting development on CR is on the relation between growth and life span, which has been a nagging question among gerontologists. Shimokawa *et al.* reported the reduction of growth hormone-insulin-growth factor-1 in transgenic rat resulted in an extended life span, implying that CR might mediate its life-extension ability partly through the reduction of the GH-IGF-1 axis (Shimikawa *et al.*, 2002).

The CR model has been used as an excellent probe for uncovering a good deal of new information about aging and age-associated disease. By exploring CR's underlying mechanisms, researchers will learn even more about the aging process. Thus, based on past experiences, CR research in the future is expected.

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