Caloric Restriction Mimetics: Metabolic Interventions

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Caloric restriction (CR) retards diseases and aging in laboratory rodents and is now being tested in nonhuman primates. One way to apply these findings to human health is to identify and test agents that may mimic critical actions of CR. Panel 2 focused on two outcomes of CR, reduction of oxidative stress and improved glucoregulation, for which candidate metabolic mimics exist. It was recommended that studies on oxidative stress should emphasize mitochondrial function and to test the efficacy of nitro TITOS and other antioxidants in mimicking CR’s effects. Studies should also focus on the long-term effects of compounds known to lower circulating glucose and insulin concentrations or to increase insulin sensitivity. Also, four other developing areas were identified: intermediary metabolism, response to infection, stress responses, and source of dietary fat. These areas are important because either they hold promise for the discovery of new mimetics or they need to be explored prior to initiation of CR trials in humans. Other recommendations were that transgenic approaches and adult-onset CR should be emphasized in future studies.

GERONTOLOGICAL studies of caloric restriction (CR) have been pursued for more than 65 years and can be clustered into three phases. The major accomplishment of the first phase showed that chronic CR delays the onset of most age-related diseases, attenuates the development of many physiological expressions of aging, and extends maximum life span in rodents (1–4). These studies were important because they provided convincing evidence that CR increases survival by retarding the rate of aging. Currently, CR is the only experimental manipulation that has been shown to consistently retard aging in rodents and to reduce disease and health risks in nonhuman primates. It is clear that the effects of CR depend on the reduction of calories per se and not upon a reduced consumption of other dietary components (5).

Importantly, the vast majority of studies on longevity and diseases in calorie-restricted rodents have initiated CR early in the life span; accordingly, far too little is known about CR started at or beyond middle age. The scarcity of data on adult-onset CR is problematic when considering direct human usage. There is evidence, however, that CR imposed at 12 months of age in mice can extend the maximum life span by 10–20% (2).

The second phase of CR research is characterized by a search for the mechanism(s) underlying the retardation of aging and diseases by CR. This is a challenging pursuit because CR induces thousands of biological changes, rendering it quite difficult to identify primary (initiating) mechanisms. Accordingly, these studies have been correlative in nature. Although correlative experiments are important because they define processes that are altered by CR and provide investigators with information that can be used to support or refute various theories of how CR retards aging, these types of experiments do not allow one to test directly the role of a specific gene or process in the actions of CR.

In the third phase, the relevance of CR to human health is being explored through studies in rodents and nonhuman primates. This application to humans could be either direct (i.e., people on long-term CR) or indirect (i.e., people treated with a drug that mimics its critical actions). Currently, CR is being tested in nonhuman primates for its influences on aging and associated pathophysiological changes (6–9). It is clear that CR (typically ~30% lowering of caloric intake) can be studied safely in monkeys. Certain actions observed in rodents [e.g., improved glucoregulation...
(10,11), lowered body temperature (12) also occur in rhesus monkeys. As for “CR mimetics,” very little work has been conducted. The CR mimetic studies would employ experimental interventions to test specific mechanisms hypothesized to underlie CR’s retardation of aging. Such studies are difficult because they require the long-term manipulation of specific processes in the intact organism; however, these explorations are necessary if we are to advance our understanding of the mechanism(s) of CR and to apply this knowledge for the improvement of human health.

Although this panel considered many viable candidate topics, it was decided to focus attention on two areas of inquiry: (a) oxidative stress and glucoregulation, for which candidate metabolic mimics exist; and (b) four areas of promising development—intermediary metabolism, response to infection, stress responses, and source of dietary fat. The developing areas are important to study in the context of CR because either they hold promise for the discovery of new mimetics or they need to be explored prior to considering human CR trials. Emphasis on these topics is placed on transgenic approaches for discovering and mimicking CR’s underlying mechanisms and also the need for more information on the influences of adult-onset CR.

**Oxidative Stress**

The role of oxidative stress of mitochondrial origin in aging is being studied extensively. Likewise, there is much interest in the possibility that CR may act to retard aging by lowering oxidative stress/damage (13). This section focuses on the influences of CR on reactive oxygen species (ROS) generation, oxidative damage, and ROS-mediated cellular signaling and apoptosis. We also consider the role that proton leak and mitochondrial membrane composition may play in regulating oxidative status.

It is possible that α-phenyl-tetra-butyl nitroxide (PBN) and other nitroxide-based free radical trapping agents (also known as spin-traps), as well as other antioxidants, will act as CR mimetics to prolong both average and maximum life span. Preliminary data that support this possibility exist; however, this notion needs to be evaluated rigorously, and this section gives emphasis to confirming and extending these observations. Further, research is proposed to understand better the influence of nitroxide compounds on the accrual of oxidative damage with aging.

**Glucoregulation**

A well-recognized consequence of CR in rodents and rhesus monkeys is a lowering of circulating glucose and insulin as well as an improvement of insulin sensitivity. Related to this is the “glycation theory of aging,” which proposes that blood glucose itself causes cell and tissue damage, known factors in aging. This section focuses on methods to pharmacologically mimic CR’s ability to reduce circulating glucose and insulin levels as well as to increase insulin sensitivity. Also considered are approaches to attenuate age-associated increases in levels of glycated and glycoxidized macromolecules.

**Transgenic Approaches**

The development of the techniques to produce transgenic animals has been an important advance because it provides a system for studying, in the whole animal, the role of individual genes in both normal physiological functions and in disease processes. Transgenic animals are a valuable resource for studying the retardation of aging by CR because they allow investigation of the effect of a specific gene (or process) on aging (14,15). In the context of oxidative stress, transgenic mice are available that overexpress genes coding for antioxidant enzymes as well as other genes involved in DNA repair of damage induced, in part, by ROS. The proposed use of genetically altered animals is confined neither to ROS mimics (e.g., transgenic mice overexpressing Glut-4 may mimic CR animals in having lower blood glucose) nor to rodents (e.g., long-lived C. elegans mutants may metabolically resemble parts of the CR phenotype).

**Developing Areas**

Panel 2 has identified four topics that are worthy of exploration in order to provide data that may guide the development of CR mimetics 5–10 years from now.

**Intermediary metabolism.**—Because one hallmark of CR is that energy input into the organism is reduced, it follows that energy metabolism may be altered. However, an overt gap in the body of knowledge on CR concerns its influence on intermediary metabolism in most organs. For example, very little data exist on the influences of long-term CR on the activities or regulation of the enzymes for most major pathways (e.g., glycolysis, gluconeogenesis, amino acid metabolism, the Krebs cycle, and fatty acid metabolism), contributing metabolites, and their concentrations and flux through various pathways. Delineation of the response of these pathways to CR may yield mimetics of CR that can be used to make specific shifts in intermediary metabolism that produce “CR-like” states.

**Response to infection.**—Many publications describe the immunological effects of CR in rodents; however, most of the studies employed in vitro testing. Because rodents on CR are typically raised in a controlled, pathogen-free environment, little is known about the ability of the immune system in CR rodents to respond to relevant pathogens following in vivo challenge. This point is important because, in the natural environment, organisms are constantly exposed to various microorganisms. Therefore, it is important that systematic studies in multiple strains of mice and rats be conducted to evaluate their resistance/susceptibility to infection, both in young and old animals subjected to long-term CR.

**Stress responses.**—It has been suggested that CR shifts an animal’s resources into maintenance functions so that it is better able to respond to a variety of environmental stresses (16). If this is true, then it is possible that the retardation of aging by CR is at least partially a result of the restricted animal being more resistant to the harmful actions of stress and toxic insults. This idea is supported by systems involved in cellular protection (e.g., heat shock response, DNA repair) being enhanced by CR. Further, most of these cellular protection mechanisms are compromised with age. There is direct evidence that rodents on CR are more resistant to a variety of damaging agents or insults such as surgi-
Dietary fat source in CR studies.—The panel recognized the importance of studies exploring the quality of the diet used in CR studies and in those attempting to mimic CR’s effects. For example, the source of dietary lipids has not been carefully studied in the context of CR. Specifically, the long-chain omega-3 polyunsaturated fatty acids (PUFAs) are well-established suppressors of autoimmunity, cardiovascular disease, and some types of cancers (20,21). Therefore, it is plausible that feeding certain types of lipids, such as the omega-3 PUFAs, could enhance the beneficial effects of CR with respect to aging. Accordingly, it seems timely to investigate the source and level of dietary fatty acids in CR studies with rodents.

Background

Oxidative Stress

Many studies have shown that aging is associated with an increase in oxidative damage to cellular macromolecules (13,22). The ROS hypothesized to be important in aging are thought to be largely of mitochondrial origin and to arise from the electron transport system (ETS) as a normal consequence of energy metabolism. One mechanism responsible for life-span extension with CR could involve reduction in ROS production. CR has been shown to inhibit or delay age-related increases in oxidative damage to proteins (23), DNA (24), and lipids (25). The cellular changes responsible for this decrease in oxidative damage, however, are still open to speculation. Oxygen free radical production is greatest during state 4 respiration (26); thus, processes that decrease state 4 respiration may lower free radical production. State 4 respiration is primarily controlled by the rate of proton leak across the inner mitochondrial membrane (27). Increasing evidence suggests that proton leak may play a major role in regulating ROS production, and a reduction in proton leak by CR may be central to the action of this treatment.

Mitochondrial proton leak.—Indirect evidence and a few direct studies suggest that proton leak may play an important role in the aging process. Proton leak, like maximum life span, has been shown to be inversely correlated with body mass among different species of mammals (28). Similarly, proton leak is lower in a long-lived reptile than in a weight-matched mammal (29). Within the animal, mitochondrial proton leak rate is also higher in brain and muscle, tissues which tend to show the highest levels of oxidative damage, than in liver or kidney (30). These results suggest a negative correlation between proton leak and maximum life span or tissue oxidative damage. A study in rat hepatocytes has shown that aging is associated with a decrease in mitochondrial membrane potential and an increase in production of ROS (31). In support of this finding, mitochondrial proton leak was observed to be higher in hepatocytes from old compared to young mice (32). Mitochondrial oxidative damage, induced by peroxynitrite, has also been shown to cause an increase in mitochondrial proton leak (33), suggesting that accumulation of oxidative damage with aging further increases proton leak. Although the mechanism of mitochondrial proton leak is unknown, membrane fatty acid composition and uncoupling proteins (UCPs) have been proposed to play a role, and both are influenced by CR (34).

It has been concluded that a low rate of ROS production contributes to a decelerated rate of aging both in animals that conform to the rate of living theory (e.g., mice, rats, dogs, cows) and in animals such as birds, which deviate from the predictions of this theory (35). Although proton leak correlates well with longevity in species that obey the rate of living theory, it is unlikely that proton leak is the only means through which the rate of ROS production and longevity are regulated. For example, it has been shown that birds have a lower rate of ROS production than do mammals of similar size, primarily because of decreased ROS formation at Complex I of the ETS (36,37). These results suggest that the structure of Complex I in birds has evolved in a manner to minimize electron leak to oxygen. It is unlikely, however, that CR could induce such a change in Complex I. Accordingly, research on CR should focus on its influences on proton leak or other processes that may lower ROS production.

Mitochondrial membrane fatty acid composition.—Several reports describe a correlation between proton leak and membrane fatty acid composition, with most finding that proton leak is positively correlated with long chain polyunsaturated fatty acids (LCFA) and negatively correlated with linoleic acid (38–40). The changes in tissue fatty acid composition with aging and CR support the idea that proton leak is increased with aging and decreased with CR. In cross-species comparisons, a low docosahexaenoic acid concentration and a low degree of fatty acid unsaturation in mitochondria are associated with a long life span in mammals (41) and birds (42). In rat heart, age-associated increases in LCFA and decreases in linoleic acid were reported, and these changes were prevented by CR (43). Similarly, studies in spleen cells (44) and liver mitochondria (45,46) found that CR prevents both an age-related decrease in linoleic acid concentration and an increase in LCFA.

It is possible that CR lowers the rate of proton leak and ROS production by altering mitochondrial membrane fatty acid composition. Treatments that mimic the changes in membrane fatty acid composition observed with CR may be useful in altering mitochondrial membrane permeability and ROS production. Thus, this research area is tightly linked to another (Source of Dietary Lipids and CR) being considered by this panel.

Mitochondrial UCPs.—In addition to the possible role that membrane fatty acids may play in regulating proton leak, the recent discovery of uncoupling proteins (UCP-2 and UCP-3) in tissues other than brown fat has offered another possible mechanism for mitochondrial proton leak (47–50). UCP-2 and UCP-3 mRNA expression is increased by fasting (51,52), whereas a short-term 50% CR resulted in a decrease in UCP-3 expression in skeletal muscle (52). Additional studies are needed, however, to determine how UCPs are regulated and the effect of long-term CR on these proteins.
Nitrones and other antioxidants.—Two studies provide evidence that PBN administration may prolong the life span of experimental animals. In the first (53), PBN (30 mg/kg) or saline was administered by ip injection to senescence accelerated mice beginning at about 20 weeks of age. Control animals (receiving saline) had a 50% survival rate of 42 weeks versus 56 weeks for PBN-treated mice. The second study (54) began PBN treatment (0.25 mg/ml in drinking water) of C57BL/6J mice at 24.5 months of age. The PBN administration continued until death. The results showed that PBN marginally, but to a statistically significant extent, increased both average and maximum life span. The average life span was 29.0 months for control versus 30.1 months for PBN-treated animals. PBN administration increased maximum life span from 31.7 by to 33.3 months but did not influence body weight.

Research on the pharmacological activity of PBN has been reviewed (55,56). Briefly, even though PBN has been used as a spin-trapping agent since the late 1960s, it was not known to have pharmacological activity until about 20 years later. PBN was first found to be effective in protecting rats against death induced by traumatic shock (57). PBN also shows neuroprotective activity in the gerbil global stroke model (58) and has efficacy even if given up to one hour after the ischemia event (59). Older gerbils are much more susceptible to a stroke than are younger ones (58). A chronic, low-level treatment of older gerbils with PBN reversed their age-enhanced susceptibility to a stroke, and this protection remained several days after cessation of PBN administration (60). Also supporting the biological activity of PBN is its ability to reverse age-related changes such as striatal muscarinic desensitization (61) and decreases in noradrenergic responsiveness (62). PBN has also been found to delay the onset of senescence in cultured human fibroblasts (63) and to inhibit early-phase liver carcinogenesis in the choline-deficient L-amino acid-defined diet model in rats (64).

What mechanism(s) underlie these diverse actions of PBN? It rapidly penetrates to most tissues and has high bioavailability (65). PBN is a potent inhibitor of gene induction caused by pro-inflammatory cytokines and/or oxidative insults. It appears that PBN can suppress enhanced signal transduction processes; for instance, PBN can prevent IL-1β activation of the p38 kinase in astrocytes (66). Its mechanism of action in this regard may derive from its ability to suppress excessive production of ROS formed by pro-inflammatory cytokines. Also, PBN suppresses production of ROS by mitochondria without altering the normal functioning of the mitochondria (67).

Nitroimidazoles and CR may modify some long-term endpoints in similar directions. For example, CR diminishes macromolecular oxidative damage. It has been known for several years that oxidation parameters are decreased by chronic PBN treatment (60,68). Caloric restriction is known to slow production of ROS from mitochondria and, as noted earlier, PBN suppresses ROS formation by mitochondria (67). Also, CR is known to depress basal body temperature, and there has been mention of this occurrence following a bolus dose of 75 and 150 mg/kg PBN (69).

Exploration of antioxidants that may mimic CR’s ability to reduce oxidative stress should not be confined to nitrones. α-lipoic acid (70,71) and coenzyme Q10 (72,73) are worthy of study as possible CR mimetics because they may reduce oxidative stress and damage. α-lipoic acid acts as an antioxidant and as a cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, which supply nicotinamide adenine dinucleotide (NADH) to NADH dehydrogenase and the ETS. Coenzyme Q10 is a component of the Q cycle of the ETS, which is thought to be the main mitochondrial pathway producing superoxide (74).

GLUCOREGULATION

The influences of CR on glucoregulation are profound and consistent in rodents and primates. The hypothesis that pharmacologic or genetic mimicry of these effects will result in signs of retarded aging is worthy of exploration.

Influences of CR on insulin and glucose levels and on insulin sensitivity.—A fairly extensive literature describes insulin and glucose metabolism in CR rodents. Plasma glucose, measured in single blood samples after various periods of fasting, is lower in restricted rats and mice compared with controls (e.g., 75,76). Significantly lower insulin and glucose concentrations measured throughout 24 hours, as well as lower HbA1c levels, have also been reported in CR versus control rats (77,78). Lower insulin levels suggest that insulin sensitivity may be increased, and this is supported by the observations of Reaven and colleagues (79). CR has also been shown to increase insulin-stimulated glucose transport in rat skeletal muscles (80,81).

More recently, glucose metabolism has been investigated in nonhuman primates subjected to long-term CR. Young adult rhesus monkeys, after only a few years on CR, exhibited fasting glucose and insulin levels that were not different from those of the controls (82). After 7 years of CR, however, fasting glucose and insulin concentrations, as well as insulin responses to glucose administration, were significantly reduced, and HbA1c levels were slightly reduced in these same animals (83). Similarly, after only one year of CR in cynomolgus monkeys, Cefalu and colleagues (9) reported no differences in fasting insulin, glucose, or HbA1c compared with controls, whereas insulin sensitivity (as determined with the insulin-modified minimal model) was significantly increased. These results agree with another study in rhesus monkeys (11).

To date, there have been no planned, systematic investigations of the effects of chronic CR in humans, partly because of the sustained and difficult change in eating behavior that would be required. The eight people who lived for 2 years in the Biosphere 2 habitat consumed a diet that was nutrient-dense, yet calorically restricted due to unexpectedly low food production (84,85). Body weight and fasting plasma glucose concentrations, total cholesterol, and blood pressure were reduced with maintenance of CR; however, values approached pre-entry levels within months of exiting the facility. In individuals with impaired glucose tolerance and diabetes, relatively short-term weight loss improves glucose tolerance (86,87). Even periods of brief CR, however, improve insulin action and reduce glucose levels prior to a detectable loss of fat mass in humans (88) and rodents.
(89). Therefore, the mechanism(s) by which acute or chronic CR improves glucose tolerance and insulin action remain unclear. A reduction in adiposity is strongly associated with improvements in glucose tolerance, but likely explains only a portion of the effect of CR on glucose metabolism.

**Glycation.**—The “glycation theory of aging” proposes that cell and tissue damage from blood glucose itself is a factor in aging. Glucose reacts with proteins, amino acids, and nucleic acids through its aldehydic form via Schiff base condensation with amino groups (90). Thereafter, it undergoes a nearly irreversible rearrangement to form Amadori products. Further, Maillard reactions ensue to slowly produce reactive and toxic covalent adducts of mostly unknown structures, called advanced glycation endproducts (AGEs). These products also can arise from reactive dicarbonyl fragments generated from glycoxidation reactions involving free glucose or early glycation products (91).

The glycation theory proposes that these modifications are significant contributors to the development of age- and diabetes-related renal, vascular, ocular, and neurological pathologies, and to aging itself. There is considerable experimental support for this hypothesis (78,92–98). For example, nonenzymatically glycated serum albumin is elevated in diabetic sera (99), and diabetic nephropathy can be induced by intravenous administration of glycated albumin (100–102). It is preferentially transported into the renal glomerulus, where it has been shown to contribute to basement membrane thickening and mesangial expansion (103,104). Further, the neurofibrillary tangles and senile plaques in brain tissue from patients with Alzheimer’s disease contain the advanced Maillard reaction endproducts pyrraline and pentosidine, whereas little if any is detected in healthy neurons of the same brain (93). Also, the Diabetes Control and Complication Trial has shown that higher mean blood glucose levels in humans correlate with a higher prevalence of diabetic retinopathy, nephropathy, and neuropathy (105).

Studies in rats treated with aminoguanidine, which inhibits formation of AGEs, indicate that interference with AGE accumulation opposes the progression of age-related cardiovascular and renal decline (98). Other age-associated changes attenuated by aminoguanidine treatment in rats include cardiac hypertrophy, nephron loss, glomerular sclerosis, albuminuria, and proteinuria.

A principal means by which AGEs exert their pathological effects is through interaction with a number of cell-surface receptors, including the receptor for AGEs (RAGE), lactoferrin, galectin-3/p60/p90, and the macrophage scavenger receptor (106–109). Among these, the RAGE appears to have a central role. It is a transmembrane, cell-surface member of the immunoglobulin superfamily (106,107,110) that is expressed on endothelium, monocytes, tissue macrophages, mesangial cells, neurons, and smooth muscle cells (111). Enhanced RAGE expression in adults is associated with pathological states such as vasculopathy, nephropathy, retinopathy, neuropathy, Alzheimer’s disease, and inflammation of vessel walls (111,112).

Binding of AGEs to RAGE clearly induces pathological changes in many target cells. For example, renal failure leads to delayed clearance of β2microglobulin and an accumulation of AGE-modified β2microglobulin. AGE-β2microglobulin binding to RAGE contributes to the initiation of inflammatory bone and joint destruction in the amyloid deposits of long-term hemodialysis patients, leading to dialysis-related amyloidosis (113,114). Binding of AGEs to the RAGE of vascular endothelium induces procoagulant tissue factor, increased permeability, and expression of vascular cell adhesion molecule 1 (115,116). There are many other examples of the association of AGE-modified serum proteins and receptor-mediated age and diabetic pathologies (117).

**Opportunities for pharmacological mimicry.**—The associations between changes of glucose metabolism accompanying CR and its longevity effects suggest the following possibilities for metabolic mechanisms: (a) lowering of glucose levels; (b) enhancement of insulin sensitivity; (c) lowering of insulin levels; (d) some combination of these.

These various aspects of glucose and insulin regulation can be affected by a number of experimental interventions, including drugs that lower plasma glucose or insulin levels, or raise insulin sensitivity. Candidate compounds include the following:

- **Sulfonylureas** (example: glyburide). These drugs work primarily by enhancing insulin secretion and pancreatic beta cell sensitivity to stimuli. An appropriate dosing regimen could lead to chronic, mild to moderate hypoglycemia in experimental animals. Insulin levels would likely be similar to those of control (but high for the circulating glucose level) or possibly mildly elevated compared with controls. Insulin sensitivity may or may not be affected in such a model.

- **Biguanides** (example: metformin). Although the exact mechanism of action is unknown, the primary effect appears to be suppression of hepatic glucose production. As this is an important determinant of fasting glucose levels, animal studies might demonstrate mild to moderate lowering of fasting glucose levels, likely accompanied by lower rather than higher insulin levels. Effects on peripheral insulin sensitivity are uncertain but unlikely to be dramatic. These drugs may have a mild anorectic effect, so caloric intake would need to be monitored and controlled.

- **Thiazolidinediones** (example: troglitazone). The precise mechanism of action of these drugs is also unknown, but they may be peroxisome proliferator-activated receptor gamma receptor agonists. Their primary effect appears to be the lowering of peripheral insulin sensitivity. Long-term treatment might result in enhanced peripheral sensitivity to insulin and some lowering of insulin levels with little effect on circulating glucose levels in otherwise normal animals.

- **Alpha-glucosidase inhibitors** (example: acarbose). These drugs work by inhibiting glucose absorption from the gastrointestinal (GI) tract by interfering with digestion of complex carbohydrates in the diet. Their primary effect is to lower postmeal glucose levels. There is no direct effect on insulin action or insulin secretion. A long-term animal model may have lower average glucose levels and lower glycosylated hemoglobin, but rela-
tively unaffected fasting glucose and little effect on fasting insulin or insulin sensitivity. Postmeal insulin levels would likely be lower. Because the drug slows the rate of glucose absorption from the GI tract, care would have to be taken to ensure that equivalent caloric intake occurred in such animals.

- **Phlorizin.** This drug lowers the renal threshold for glucose excretion, thereby increasing glucose loss. Glucose levels in experimental animals would likely be lowered, without direct effects on insulin sensitivity. Urinary glucose loss would have to be matched by increased caloric intake to maintain an appropriate caloric balance.

- **Vanadate.** Administration of vanadate to experimental animals has been shown to improve insulin sensitivity, but the mechanism is not certain. Vanadate is toxic to humans, so it is not a candidate for human translation work. However, studies in animals could test the hypothesis regarding the effect of lowering insulin sensitivity on longevity.

### Transgenic Approaches

With the advent of recombinant DNA technology and the ability to genetically engineer mice, investigators now have an experimental system whereby a specific gene or process that is altered by CR can be manipulated in rodents, and the effect of this transgenic alteration on the survival and pathology can be studied. Transgenic/knockout animals are a particularly valuable resource in studying the retardation of aging by CR because they allow an investigator to conduct specific interventions to probe more directly the biological mechanism(s) responsible for the increased longevity and decreased pathology observed with CR. However, a caveat to consider in the use of genetically altered rodents is the need to determine what effects other than the expected ones may be induced by manipulation of a single gene.

By definition, transgenic/knockout animals carry either a fragment of foreign DNA stably integrated into the genome or have a portion of the genome deleted or mutated. In general, these animals are produced so that the genetic alterations are stably transmitted to the progeny through the germ line. The first report describing the production of transgenic mice was published in 1980 (118). Subsequently, transgenic/knockout mice have been used extensively to study a variety of biomedical questions (119–124).

With conventional research models it is difficult, if not impossible, to determine the importance of a specific gene in the context of CR because of the global nature of CR: A large number of processes change with aging, and most of these are altered by CR. Two general experimental approaches can be taken with transgenic/knockout mice to investigate CR. These are: gain in function (transgenic), and reduction or loss of function (knockout). Currently, transgenic/knockout mouse models are available for studying the role of hormones (glucocorticoids and growth hormone), plasma glucose levels, oxidative damage, and genome stability in CR and aging.

In using transgenic/knockout mice for aging studies, an important consideration is the strain of mouse used. Unfortunately, many of the strains of mice currently used to produce transgenic mice have not been used in aging research. According, these strains are uncharacterized with respect to survival and disease patterns. However, this limitation can be circumvented by selecting strains of mice that are widely used in aging research. For example, transgene constructs can be injected into the fertilized eggs from inbred strains of mice to produce transgenic mice that overexpress a transgene. In addition, knockout mice containing mutated genes can be backcrossed to inbred strains of mice producing congenic lines of the transgenic mice that are nearly identical to the parent inbred strain.

A second limitation of the current transgenic/knockout mouse models is that the gene of interest is over- or under-expressed throughout the animal’s life span. Among other problems, this situation makes it difficult to separate the effects that a specific gene has on embryological development from those that it may exert on the subsequent maturation and aging of the animal. It is not possible with these methods alone to alter the expression of genes in an age-dependent fashion or to assess the effects of these alterations on the aging process. This capacity would be of particular interest in mimicking CR. Because they offer the opportunity to activate or inactivate the expression of genes at will, exogenously regulatable promoter systems, particularly when used in combination with traditional transgenic or gene knockout approaches, provide a new and potentially powerful tool for studying the role of selected genes in the retardation of aging by CR. Currently, the tetracycline-regulatable promoter system, the RU486-inducible promoter system, and the ecdysone-inducible promoter system have been used to exogenously regulate the expression of transcriptionally linked genes in transgenic mice (14). Using temporarily regulatable promoter systems, it will be possible to manipulate transgene levels throughout the life span of a rodent in a way that is comparable to CR (e.g., reduced levels in early life and enhanced levels in later life).

### Developing Areas

Panel 2 has identified four topics that are important to investigate for guiding the development of CR mimetics 5–10 years from now.

**Intermediary metabolism.**—Although the effects of starvation and diabetes on intermediary metabolism have been well described, little is known about the effects of long-term, nonmalnutritional CR regimens on specific metabolic pathways. Indirect evidence suggests that CR alters the flux of intermediates through the glycolytic, gluconeogenic, and nitrogen-metabolizing pathways. These data involve measurements of the levels of the mRNAs and the activities of enzymes catalyzing individual steps in the metabolic pathways. However, the levels of the intermediates in the pathways have not been measured. Neither have the rates nor equilibrium constants for steps of the pathways been determined.

As discussed above, changes induced by CR in carbohydrate metabolism may, in part, retard the rate of aging. It is notable that in only one multicellular organism have lifespan-regulating gene systems been partially elucidated at the molecular level. In *C. elegans*, insulin receptor signaling ap-
pears to have a central role in aging. The insulin receptor homologue of *C. elegans*, daf-2, acts on daf-16, an HNF-3/forkhead transcription-factor family member, to alter energy metabolism and development (125). In mammals, insulin may also mediate some of its actions by altering the activity of HNF-3 (126).

CR may alter the characteristics of energy metabolism while changing the flux of substrates through the major metabolic pathways. Respiratory quotient (RQ) data from CR rodents first indicated that, immediately after feeding, values approach 1.0, suggesting carbohydrates are being metabolized. RQ values remain at these levels for short times and then drop to levels suggestive of lipid metabolism (127). Conversely, in the ad lib-fed (AL) animals, values remain intermediate and fluctuate minimally, suggesting a mixed metabolism of substrate. These changes correlate to activation and inactivation patterns of pyruvate kinase (PK). In CR animals, PK is maximally dephosphorylated (activated, producing a reduction in substrate concentration that produces half-maximal velocity [K\text{m}, also known as Michaelis constant]) at the time of food consumption as the glycolytic pathway is activated and RQ approaches 1.0 (128–130). PK is rapidly phosphorylated (inactivated, increasing K\text{m}) as RQ goes toward 0.8, while the glycolytic pathway is inactivated and lipids are metabolized. In AL animals, K\text{m} increases significantly.

During aging, levels of glucose 6-phosphate dehydrogenase (G6PDH) increase; however, PK becomes resistant to allosteric activation (131–133). Conversely, in CR rodents, the amount of enzyme is lower but resistance to allosteric activation is relieved. As a result, the ratio of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to its oxidized form (NADP) is significantly higher in liver from CR animals (134). Similar changes in enzyme systems have been noted in glycogen metabolism of rhesus monkeys on CR. Basal muscle glycogen synthase (GS) fractional activity is 4–5 times higher than in AL monkeys, whereas glucose 6-phosphate (G6P) content is lower in the CR monkeys, and glycogen content was unaffected by diet (135). Similar to the findings from rodent CR studies, the GS molecule was found to have a high affinity for G6P in adult rhesus monkeys with very lean body composition [10% body fat; (136,137)]. A GS molecule with a high affinity for the allosteric activator G6P would ensure normal glycogen stores by “pulling” G6P into glycogen.

Measurements of the levels of the mRNAs and enzymatic activities of key steps in intermediary metabolism in CR rodents suggest that, in the liver, the enzymatic capacity for glycolysis is reduced and the enzymatic capacity for gluconeogenesis is increased (138,139). Studies of the effects of CR on the expression of key hepatic glycolytic, gluconeogenic, and nitrogen-metabolizing enzymes during the feeding and postabsorptive phases in mice suggest that the enzymatic capacity for gluconeogenesis is enhanced and that for glycolysis is suppressed continuously in the liver of CR mice, even after feeding (139).

However, these data are suggestive at best. The fluxes of intermediates through these pathways have not been measured. The levels of key intermediates of the pathways in vivo have not been measured. The effects of CR on the K\text{m} and V\text{max} of key steps in the pathways are unknown. Until such data are available, understanding of the effects of CR on metabolism will remain limited.

**Response to infection.**—Caloric restriction is known to enhance T-cell mediated immune function in both mice and rats through the maintenance of naive T cells (140,141). However, many questions need to be addressed in order to determine the applicability of CR to humans. These include:

- What effect does CR have in young animals? During early life, CR may decrease immune function in rodents and primates (2,142). Both short- and long-term CR may decrease humoral immune function for T-cell dependent and independent antigens. Suppressed humoral immune function in CR rodents is linked to decreased B-cell and/or macrophage function (143).
- Can rodents on CR initiate an appropriate response to bacterial infection? Whether resistance remains intact or is compromised has been examined (144,145), but further study is needed. To date, the majority of studies in vitro have focused on general T-cell function, but the modulation of antigen presenting cells, particularly macrophage antigen processing and/or phagocytosis, has not been examined. Furthermore, the influence of CR on the specific type of T-cell response (i.e., Th-1 or cell-mediated and Th-2 or antibody-mediated) has not been thoroughly studied. CR does increase or prevent the loss of naive T cells (141,146,147); therefore, the ability to initiate an immune response may not be negatively affected.
- Are the CR-fed mice able to appropriately terminate an immune response? A youthful, general T-cell function is maintained in old CR mice relative to their control counterparts, as noted by enhanced T-cell proliferation in vitro. However, the negative feedback signals that end the response appropriately may be impaired. This is significant because an immune response generates ROS as well as a catabolic physiological state.

**Stress responses.**—One proposed action of CR is to shift resources into maintenance functions, so that an animal may better respond to a variety of environmental stresses (148). Therefore, it is logical to propose that the retardation of aging by CR is at least partially a result of the restricted animal being more resistant to the harmful actions of stress and toxic insults. There is evidence that CR rodents are more resistant to a variety of damaging agents or insults including surgical trauma (17), heat shock (18), and the toxicity of a variety of drugs (19). This increased resistance in CR rodents may be a result of systems involved in cellular protection being enhanced by CR [e.g., DNA repair, protein degradation/turover, the glucocorticoid system, etc.; (2,17)]. This general idea is also supported by invertebrate studies establishing that long-lived mutants also show increased resistance to a variety of types of stress (149).

**Dietary fat source in CR studies.**—In recent years, consumption of omega-6 dietary fats has been increasing in an attempt to lower saturated fat intake and thereby to pro-
tect against cardiovascular disease (150–152). Surprisingly, omega-6 lipids have been found to be pro-inflammatory, thereby increasing the susceptibility to autoimmune disease and certain types of cancers (20). In studies using omega-3 PUFAs from plant or marine oils, pro-inflammatory cytokines are lower, and the incidence of autoimmune renal disease and cancer is reduced (21). Thus, the source of lipid and level of calories appear to be major determinants of survival in autoimmune-prone mice.

It is unknown, however, whether CR mice or rats from long-lived strains, which are not prone to developing autoimmunity, will also live longer on diets enriched in fish oil. Several studies indicate that the omega-3 PUFAs suppress lymphocyte cytokine production (153,154), signal transduction (153,155), and gene expression (156) in young, healthy C57BL/6 mice, suggesting that similar positive effects could be observed. Therefore, it is important to evaluate rigorously the source and level of dietary fat in the context of CR.

**Recommendations for Research**

**Oxidative Stress**

**Rodent Studies**

Mitochondria.—Studies testing the hypothesis that changes in mitochondrial membrane fatty acid composition are required for life-span extension with CR are important to pursue. This could be tested by experiments designed to feed rodents purified diets with different fatty acid compositions. Through dietary manipulation, it should be possible to mimic CR-related changes in membrane fatty acid composition without restricting total caloric intake. Shorter term studies could also test the hypothesis that changes in mitochondrial membrane fatty acid composition are necessary for changes in mitochondrial proton leak and rate of free radical production with CR.

Studies designed to test the hypothesis that CR results in a sustained decrease in tissue UCP-2 and UCP-3 concentrations would also be important to pursue. Experiments designed to determine proton leak through simultaneous measurement of mitochondrial oxygen consumption and membrane potential along with measurements of UCP-2 and UCP-3 concentrations should be encouraged, as the role of UCPs in regulating proton leak has not been firmly established. Studies examining the relationship between proton leak and rate of production of ROS in mitochondria isolated from CR and control animals are also timely. In a broader sense, experiments aimed at clarifying the mechanisms underlying age-associated increases in mitochondrial ROS production as well as the attenuation of this increase by CR appear to be important to pursue.

Much of the work in this area has employed methods that are indirect and only infer changes concerning the parameter of interest. For example, many studies attempt to determine proton leak rate by measuring either membrane potential or state 4 respiration. However, this approach is not optimal because proton leak rate is a function of both membrane potential and respiration rate and should be determined by simultaneously measuring both respiration and membrane potential. Many UCP studies also rely on measurements of mRNA levels. These studies do not directly determine UCP concentrations in mitochondrial membranes or the effects of these proteins on membrane permeability. It is important to directly measure UCP concentrations in isolated mitochondria and to combine these measurements with determinations of mitochondrial proton leak whenever possible.

Studies examining the effects of CR or other dietary manipulations on tissue fatty acid compositions should avoid methods that measure fatty acids in homogenized whole tissues or methods that do not provide pure isolations of subcellular organelles. Methods that isolate specific membranes (plasma membrane, inner mitochondrial membrane, outer mitochondrial membrane) would be especially appropriate for this type of study.

Nitrene and other antioxidants.—The background information presented above builds a compelling case for the need to test the hypothesis that PBN or other spin trap compounds reduce the age-associated accrual of oxidative damage and increase maximum life span. The experiments should have a positive CR control and a proper negative control that is pair-fed to the caloric intake of the treated animal. Additional experiments could be performed to determine if spin trap compounds cause changes in body temperature, energy expenditure, blood glucose concentrations, and other physiological or biochemical measurements similar to changes observed with CR. Determining optimal doses and how they may differ among animal models will be critical.

Transgenic approaches.—Investigation of transgenic rodents should provide important opportunities to determine the role that lowered oxidative stress/damage may play in CR’s retardation of aging. Using transgenic mice that either overexpress or underexpress various antioxidant enzymes (e.g., Cu/Zn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase), it is potentially possible to study specifically the role of oxidative damage in the actions of CR (15). This type of experimental manipulation has indicated that the lowering of oxidative damage by overexpressing genes for antioxidative enzymes plays a role in aging in *Drosophila* (157). The panel views studies of genetic modifications leading to a lowering of oxidative stress and damage worthy of pursuit.

Although there are many types of mutagens, it is well known that ROS can damage DNA and cause mutations. It has been suggested that CR extends the longevity of rodents and reduces the incidence of age-related pathological lesions by reducing the levels of DNA damage and mutations that accumulate with age. For example, the incidence of cancer and other pathological lesions, which are associated with changes in DNA damage/mutations, is reduced or retarded by CR, and cells from rats fed a CR diet show enhanced DNA repair and reduced levels of DNA damage/mutations (158). Recently, investigators in the area of DNA repair have generated a variety of transgenic mice deficient
in various DNA repair proteins and pathways. These mice would be valuable in studying the role of DNA damage and mutations in aging. Arguably, it is more important to generate transgenic mice that show enhanced DNA repair and to determine if reducing DNA damage/mutations results in increased survival and pathology as observed in CR.

Nonhuman Primate Studies

Studies testing the hypothesis that CR increases linoleic acid concentrations and decreases long chain polyunsaturated fatty acids (C22:4, C22:5, and C22:6) in subcellular membranes would be helpful. The rodent studies of membrane fatty acid changes with CR have not been confirmed in other species. Studies to test the hypothesis that CR decreases proton leak also need to be completed in a primate species. Very little is known about changes in mitochondrial respiration and membrane potential following CR in a primate species; any functional mitochondrial measurement in these species would provide valuable new information.

Human Studies

Certain rodent and nonhuman primate studies should be completed before human studies are considered.

In Vitro Studies

Because assay of mitochondrial respiration and membrane potential requires isolated cells or mitochondria, the studies discussed above contain an in vitro component. Cells in tissue culture (including genetically modified lines) may also be used to test the relationship among mitochondrial fatty acid composition, proton leak, and rate of ROS production.

Glucoregulation

Research is needed to test the hypothesis that lowering of circulating glucose and an improvement of insulin sensitivity will mimic fundamental actions of CR in rodents and rhesus monkeys. Because interventions that affect glucose levels may also affect insulin sensitivity and vice versa, research recommendations for these two potential mediators of CR effects will be discussed together. However, one important goal of such research is to segregate and define the specific contributions of glucose lowering and enhanced insulin sensitivity.

Lowering Levels of Circulating Glucose and Insulin; Increasing Insulin Sensitivity

Rodent studies.—Based on the availability of the pharmacologic agents previously described, a series of long-term intervention studies could be carried out in rodents to affect circulating glucose levels, insulin sensitivity, and circulating insulin levels in a variety of ways. Particular emphasis should be placed on elucidating the precise mechanisms of action of these agents, followed by more focused studies on those agents that best mimic the glucoregulatory effects of CR. Specific studies could be designed to test the hypothesis that these agents increase longevity in rodents. Studies also should be designed for rodent species to test the hypothesis that these compounds mimic CR effects primarily by glucose lowering or, secondarily, by affecting other metabolic or neuroendocrine pathways regulating candidate biomarkers such as reduced body temperature.

Transgenic mice overexpressing GLUT4 have been produced (159) that show lowered levels of plasma glucose resembling those observed in CR mice. These mice provide an excellent model for studying how lowered plasma glucose levels affect glycation and glycoxidation reactions and aging. Use of transgenic approaches to modify insulin signaling pathways can help define the importance of insulin resistance for CR effects.

Nonhuman primates.—Subsequent work should be carried out in nonhuman primates, guided by the results of rodent studies. Agents that affect longevity of rodents would be candidates for testing in nonhuman primates. Regardless of effects of these agents on longevity and related markers in rodents, studies in nonhuman primates should test their effects on age-related disease outcomes such as diabetes, hypertension, and atherosclerosis.

Glycation

Because the glycation theory of aging is untested using life-span extension as an endpoint, at least three approaches to reducing glycation damage should be considered for these studies. First, as just discussed, research should be supported using agents that result in reduced blood glucose levels. Second, studies should be conducted to develop and test agents that block or reverse glycation damage. Aminoguanidine is the prototype of a glycation-blocking agent, but has broad effects and may be nonspecific. Other agents, which disassociate the cross-links in AGEs, have been described (160). Further development of such agents, and their effects on life span, should be explored. Third, the development and testing of agents that interfere with the interaction between AGEs and their receptors may be effective in opposing the development of age-related pathologies that arise from glucose-damaged proteins (116,161).

Possible genetic approaches to examine components of the glycation pathways should be pursued as further understanding of specific genes controlling these pathways is elucidated. Similarly, genetic approaches could be applied to regulation of the interaction of AGEs with their receptors and to test the hypothesis that increasing protein turnover could mimic the effects of CR by reducing glycation.

Developing Areas

Intermediary Metabolism

Studies should be supported that investigate the hypothesis that changes in the regulation of energy metabolism are involved in the mechanism by which CR extends life span. Initially these studies should delineate the way in which CR modifies carbohydrate, amino acid, and lipid metabolism. The ETS is worthy of close examination. The levels of intermediates in the pathways should be determined. Where possible, the rates and equilibrium constants of the reactions should be determined as should the levels of allosteric modifiers of key reactions. The specific activity of selected key enzymes should be studied. Where applicable, the studies
should include investigation of the specific activity and post-translational modifications of the enzymes.

Future goals of the studies should be to delineate the mechanism(s) by which alterations in metabolism result in life-span extension. These studies could, for example, involve approaches for altering regulation of intermediary metabolism in ways that mimic CR. These approaches may involve the identification and development of activators/inducers of specific enzyme systems that produce desired flux through specific pathways. Proven approaches that have been successfully employed to study the effects of diabetes on intermediary metabolism would be useful. Also, the development of genetic means to accomplish these goals should be encouraged.

Response to Infection

New rodent studies with caloric intake restricted by 10% or 20% should be undertaken, which would be more applicable to humans than results from moderate to severely restricted diets. It has not been established whether CR-fed mice differentially express MHC class I and II molecules, which are distinctly recognized by CD4 and CD8 T cells. It is well established that the level of MHC class I molecule expression determines functional activation of cytotoxic T cells against intracellular pathogens, whereas MHC class II engagement by CD4+ T cells on B cells activates them to produce antibody against extracellular pathogens. Therefore, systematic studies in CR-fed mice infected with selected intr- and extracellular pathogens common to humans need to be pursued. These studies should involve examination of the magnitude and duration of immune response as well as microbial clearance from the animal. Additionally, the key immune players, T cells and antigen-presenting cells such as B cells and macrophages, should be analyzed to determine Th-1/Th-2 cytokine responses and MHC class I and II expression, respectively. These experiments would give insight into which arm(s) of the immune system are most affected by CR and how it relates to clinically relevant infections.

Stress Responses

In an extension of the type of studies proposed for response to infection, it is important to determine the in vivo “toughness” of restricted rodents to a variety of stressors. This would include thermal challenges, responses to injected tumor cells, and the ability to withstand cellular damage induced by selected toxins. Such studies should evaluate not only the resistance of the whole animal, but also determine molecular and cellular mechanisms that underlie differences in stress responses observed in CR animals.

Dietary Fat Source in CR Studies

Studies to explore the influence of different levels and types of dietary fats in the context of CR are needed. The current recommended dietary fats for humans is 30% of total calories, of which 10% or less is saturated fat and the remaining is a mixture of monounsaturated and polyunsaturated fatty acids.

In the case of animal studies, generally one type of dietary oil that typically varies from 5% to 20% of diet weight is included. Diets containing high or low fat, when fed either ad libitum or in calorically controlled amounts, may produce marked differences in body weights and, possibly, survival. Further, to better mimic human diets, it will be necessary to have a mixture of saturated, monounsaturated, and polyunsaturated lipids in animal diets. To achieve the level of fat in human diets, it will be necessary to increase dietary fat in animal feeds to ~15% and to use a mixture of two to three dietary oils. Also, because the derivatives of specific fatty acids can have bioactivity (162), it is important to use diets with defined levels of fatty acids.

Recommendations on Methodologic Issues

The panel has identified seven methodological topics which are important to consider in pursuing these lines of research:

- Improved markers for quantitation of oxidative damage and glycation.
- Conduct of research which couples the effects of a CR mimetic to data on longevity and disease patterns.
- Methods to quantify insulin sensitivity and secretion (especially in rodents).
- Need to match the caloric intake of control animals to that of subjects treated with a CR mimetic.
- Development of time-regulatable transgenic systems.
- Detailed protein characterization, identification of intermediates, and direct measurements of fluxes through the pathways of intermediary metabolism.
- Use of activators/inducers/inhibitors of specific enzyme systems as well as transgenic means to alter fluxes through specific metabolic pathways.

Recommendations for Resources Not Available for Investigator-Initiated Projects

The panel has identified three categories of essential resources needed to conduct the indicated research.

High-quality reagents and supplies.—These include nitrones, antioxidants such as lipioic acid and the glucoregulatory-mimetic drugs; antibodies to AGEs and other markers of age-associated molecular damage; microarray chips for study of gene expression.

Rodents.—Animal facilities are required to allow measurement of longevity and pathology under appropriately controlled conditions. Expanded study of adult-onset CR and adult-onset CR mimetic interventions as well as intermediary metabolism would also contribute to this resource need.

Nonhuman primates.—The panel viewed the continued support for the current CR colonies as a very high priority. Also, support of collaborative studies of these existing animal resources (including users and hosts) is needed. Finally, there is a need to expand CR and control colonies to allow for the study of animals not currently being investigated for effects of CR on life span. These cohorts are required to allow for more invasive studies and tissue banking.

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