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Caloric restriction and redox state: Does this diet increase or decrease oxidant production?

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Calorie restriction (CR) is well established to enhance the lifespan of a wide variety of organisms, although the mechanisms are still being uncovered. Recently, some authors have suggested that CR acts through hormesis, enhancing the production of reactive oxygen species (ROS), activating stress response pathways, and increasing lifespan. Here, we review the literature on the effects of CR and redox state. We find that there is no evidence in rodent models of CR that an increase in ROS production occurs. Furthermore, results in *Caenorhabditis elegans* and *Saccharomyces cerevisiae* suggesting that CR increases intracellular ROS are questionable, and probably cannot be resolved until adequate, artifact free, tools for real-time, quantitative, and selective measurements of intracellular ROS are developed. Overall, the largest body of work indicates that CR improves redox state, although it seems improbable that a global improvement in redox state is the mechanism through which CR enhances lifespan.

Keywords: Calorie restriction, Reactive oxygen species, Free radicals, Hormesis, Aging

Introduction

It is well recognized that, overall, aged animals present higher levels of oxidatively modified proteins, lipids, and nucleic acids.^{1–4} However, many genetic interventions (such as removing antioxidants) leading to overall enhanced oxidative damage have little or no effect on lifespan. In addition, antioxidant supplementation does not generally have a beneficial lifespan-enhancing effect. Furthermore, many long-lived organisms present significant oxidative modifications. Overall, these findings, lead to the conclusion that accumulation of oxidative damage to biomolecules *per se* is not sufficient to limit lifespan.^{5,6}

On the other hand, some specific genetic interventions that affect redox modifications of biomolecules, such as the removal of specific DNA repair enzymes, promote lifespan limitation, and premature aging.⁷ In addition, at least one specific intervention that prevents oxidative modifications, the expression of catalase in mitochondria, increases animal lifespan.⁸ This leads to the hypothesis that specific and yet unidentified modifications of biomolecules may be determinant in aging, although overall redox state seems not to be.

Caloric restriction (CR), or the limitation of ingested calories without malnutrition,^{9,10} is well documented to extend lifespans in a wide variety of

organisms, ranging from yeasts to rodents. The literature produced up to 10 years ago examined the effects of CR on redox state in quite a detailed manner, and came to the conclusion that this dietary intervention decreased age-associated oxidative damage, as will be detailed below. This conclusion was based on a variety of observations, including measurements of oxidized protein, DNA, and lipids in aged rodents on *ad libitum* versus CR diets, measurements of isolated mitochondrial release of oxidants from these animals, and the quantification of high- and low-molecular-weight antioxidants.

On the other hand, a group of researchers recently proposed a radically different idea: that CR enhances the generation of oxidants, therefore activating stress response pathways that lead to a prevention of aging.^{11–15} Indeed, it has been proposed that increased oxidative stress, by promoting hormetic responses, mechanistically integrates a large number of interventions capable of extending lifespans.¹⁵ While this view is well in line with the growing recognition of the importance of reactive oxygen species (ROS) as signaling molecules, it is difficult to reconcile with the massive experimental evidence that CR decreases oxidative damage to biomarkers with age and the generation of oxidants from isolated mitochondria in animals, as described below.

Many factors differ between the experimental evidence cited by these newer studies suggesting that

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CR increases oxidation and earlier studies suggesting that CR improved redox state: (1) initial studies focused mostly on rodents, while more recent studies include CR models in a variety of model organisms, including *Caenorhabditis elegans* and *Saccharomyces cerevisiae*; (2) dietary interventions have varied greatly in more recent years in rodent models¹⁰ and also vary significantly between different organisms; (3) early studies used mostly modified biomolecules as tissue markers of redox state, while today many groups attempt to measure ROS levels within cells and tissues, often using fluorescent probes as a methodological approach. We will examine each of these points in an attempt to clarify the current understandings regarding the effects of CR on redox state.

Effects of CR on redox state in less complex model organisms

Using *C. elegans*, Schulz *et al.*¹² determined that an intervention that mimics CR, the presence of the inhibitor of glycolysis deoxy-glucose, promoted an increase in lifespan accompanied by enhanced respiratory activity. In parallel, the formation of ROS in the tissues was monitored using dichlorofluorescein (DCF, see discussion below), which showed enhanced fluorescence in the presence of deoxy-glucose. This evidence, added to the observation that antioxidants prevented the effect of deoxy-glucose, led the authors to propose that CR enhanced lifespan in *C. elegans* by increasing oxidative stress.

Ralser and Benjamin¹⁶ have argued that the damaging antioxidant effects observed in this publication could be a result of reductive stress, and that further experiments are necessary to clarify the effects of deoxy-glucose on *C. elegans* lifespan.

Even if dietary restrictions do indeed enhance oxidant production in *C. elegans*, this is far from strong evidence that similar mechanisms are present in more complex animals. Indeed, aging in *C. elegans* is quite unique. A hallmark of a restrictive diet in this organism is the acquisition of the metabolically depressed dauer form, which extends survival.¹⁷ Furthermore, many genetic interventions that inhibit mitochondrial respiration in *C. elegans* extend lifespan,^{18–20} while even partial respiratory inhibition is causative of age-associated disease in vertebrates (reviewed in Lin and Beal²¹).

Drosophila melanogaster is an organism in which antioxidant interventions have an impact on survival, and even overall redox state seems to be more related to lifespan.⁵ In *D. melanogaster*, dietary restriction does not change isolated mitochondrial ROS release,²² but decreases lipid oxidation²³ and age-related accumulation of oxidized proteins.²⁴

In *S. cerevisiae*, the effects of CR on redox state are controversial. Lin *et al.*²⁵ found that glucose

restriction in this yeast did not increase the expression of a panel of antioxidant enzymes or result in increased resistance against exogenous oxidants, and we also find²⁶ that resistance to exogenous H₂O₂ is unaltered by CR. On the other hand, the expression of specific antioxidants is enhanced: the highly effective H₂O₂-removing peroxiredoxin²⁷ and superoxide dismutases.^{14,28,29}

Some authors^{14,28,29} have proposed that CR enhances cellular oxidant production in *S. cerevisiae*, resulting in a hormetic response that promotes protection against aging. Mesquita *et al.*²⁹ have specifically proposed that CR increases H₂O₂, enhancing antioxidant defenses, and protecting against the effects of more reactive superoxide radicals. This hypothesis is largely based on intracellular ROS measurements using dihydrorhodamine 123 (DHR²⁹) and DCF,^{28,29} which unfortunately present very serious methodological caveats, in particular in the *S. cerevisiae* model, as will be described in detail below.

Indeed, this idea is in direct opposition to measurements of H₂O₂ release from isolated mitochondria or permeabilized cells^{26,30} that found that CR in yeast decreases H₂O₂. In addition, CR in *S. cerevisiae* significantly protects against glutathione oxidation in young cells^{26,31,32} and also decreases protein carbonylation.^{32,33} Altogether, these data suggest CR in yeast prevents ROS accumulation, leading to lower levels of protein oxidation during chronological aging.

Overall, some data using simpler eukaryotic model systems exist suggesting that CR may increase ROS production and lead to a hormetic response; however, an equal body of work suggests the opposite. As a result, the overall effect of CR on redox state in these organisms is still far from reaching a consensus. A critical problem in this respect is the lack of methodologically sound *in vivo* measurements of real-time production of specific oxidants, as will be discussed subsequently.

Effects of CR on redox state in vertebrates

A vast number of publications demonstrate that CR in laboratory rodents decreases tissue levels of oxidatively modified lipids,^{34–40} proteins,^{41–46} and DNA.^{47–50} A pronounced effect of CR is to decrease oxidative damage of mitochondrial components that occur as animals age.^{51–53}

Another large body of work demonstrates that ROS release from mitochondria or tissues from CR animals is decreased.^{37,53–67} Other publications found no changes in ROS production with CR^{46,68–71} and many propose that CR-induced decreases in ROS release depend on tissues examined, time on the diet, animal age when the diet was initiated, and gender. No experimental publication was located

demonstrating that CR in rodents enhances ROS generation in non-inflammatory tissues.

It should be noted that the evidence listed above is specific to CR, or the limitation of dietary calories without malnutrition. In more recent years, many dietary interventions that are not identical to CR have been adopted, and are often called 'caloric restriction'.¹⁰ Many of these diets are in fact the restriction of total food, incurring malnutrition, and may lead to a loss of tissue antioxidant capacity.⁶⁷ Other researchers have adopted intermittent or every other day feedings as 'caloric restriction' protocols.⁷² We recently found that this feeding pattern very significantly increases ROS release from skeletal muscle and adipose tissue, while CR prevents this release.⁶⁷ Indeed, unlike CR, fasting has previously been shown to increase ROS release.⁷³ Thus, from a redox standpoint, it is critical to consider the specific diet adopted.

Some publications have found increments in the activities of specific antioxidant pathways with CR,^{35,74–79} although determining the effects of CR on tissue antioxidant capacity is complicated by the vast diversity of these systems in rodents, and by the fact that expression of antioxidant enzymes is often not directly related to their activity.

Overall, in rodents there is strong evidence that CR prevents oxidative modification to tissues, some evidence that antioxidant capacity may be increased and multiple reports of decreases in the release of oxidants from mitochondria or tissues.

Measuring ROS levels in cells and tissues – methodological issues

To understand how CR prevents oxidative damage to tissues in view of the two proposed mechanisms (decreased ROS production versus increased ROS production leading to a hormetic response), measuring levels of specific ROS *in situ* within cells and tissues is critical. Unfortunately, this necessity is marred by methodological difficulties in measuring ROS, which present enormous chemical diversity and intricate reactivity within the biological complexity of the intracellular environment.

The studies in model systems that measured increases in ROS release with CR used DCF as a probe to measure oxidants within cells or organisms.^{12,28,29} DCF is a non-selective fluorescent probe that reacts with nitric oxide (NO[•]) and peroxynitrite, in addition to H₂O₂ and other ROS.^{80,81} This lack of selectivity is critical, since CR has been recently shown to increase NO[•] signaling, a central regulator of mitochondrial biogenesis.^{72,82} Furthermore, DCF is very sensitive to changes in pH within the physiological range,⁸³ and fluorescence increases in more alkaline environments. This is especially important

in the *S. cerevisiae* CR model, since these cells are grown in poorly buffered media, and undergo a large metabolic shift when incubated under control or CR conditions. *S. cerevisiae* is a Crabtree-positive yeast that, when cultured in high glucose, presents predominantly fermentative metabolism and undergoes respiratory de-repression when cultured under CR, an effect central toward lifespan expansion.⁸⁴ As a result of lower fermentative activity, CR cells present pronounced increases in pH compared to control cells (up to 3 pH points, Tahara and Kowaltowski, unpublished results), which can, alone, explain large increases in DCF fluorescence in this model even in the absence of changes in redox state.

Another probe used to measure ROS *in situ* in *S. cerevisiae* CR was DHR.²⁹ The oxidized product of DHR is positively charged, and used as a mitochondrial membrane potential marker.⁸⁵ Since CR in *S. cerevisiae* significantly increases mitochondrial activity,^{25,26,84} the accumulation of this dye in CR cells may be attributable to enhancement of mitochondrial density and membrane potentials, and not to increased H₂O₂. In addition, DHR is not a selective probe for H₂O₂,⁸⁰ as claimed by the authors.²⁹ As a result, the conclusion that CR increases ROS, specifically H₂O₂, based on the use of these probes is questionable.

Conclusion

Although it seems clear that hormetic responses exert a variety of positive cellular effects, the evidence that CR promotes an increase in intracellular ROS triggering a hormetic response and resulting in enhanced lifespan is unconvincing.

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