Research article

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 models[10] 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 Benjamin[16] 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 Beal[21]).

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 oxidation[23] 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 find[26] that resistance to exogenous H_2O_2 is unaltered by CR. On the other hand, the expression of specific antioxidants is enhanced: the highly effective H_2O_2-removing peroxiredoxin[27] 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 H_2O_2, 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 (DHR[29]) 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 H_2O_2 release from isolated mitochondria or permeabilized cells[26,30] that found that CR in yeast decreases H_2O_2. In addition, CR in *S. cerevisiae* significantly protects against glutathione oxidation in young cells[26,31,32] and also decreases protein cabonylation.[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 ($NO^{\cdot}$) and peroxynitrite, in addition to H_2O_2 and other ROS.[80,81] This lack of selectivity is critical, since CR has been recently shown to increase $NO^{\cdot}$ 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 H_2O_2. In addition, DHR is not a selective probe for H_2O_2,[80] as claimed by the authors.[29] As a result, the conclusion that CR increases ROS, specifically H_2O_2, 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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References

1 Ames BN. Endogenous oxidative DNA damage, aging, and cancer. Free Radic Res Commun 1989;7:121–8.
2 Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. Science 1996;273:59–63.
3 Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 1997;272:20313–6.
4 Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000;408:239–47.
5 Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. Free Radic Biol Med 2007;43:477–503.
6 Hekimi S, Lapointe J, Wen Y. Taking a 'good' look at free radicals in the aging process. Trends Cell Biol 2011;21:569–76.

7 Diderich K, Alanazi M, Hoeijmakers JH. Premature aging and cancer in nucleotide excision repair-disorders. DNA Repair (Amst) 2011;10:772–80.
8 Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, *et al.* Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 2005;308:1909–11.
9 McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. Nutrition 1989;5:155–71.
10 Cerqueira FM, Kowaltowski AJ. Commonly adopted caloric restriction protocols often involve malnutrition. Ageing Res Rev 2010;9:424–30.
11 Tapia PC. Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: 'Mitohormesis' for health and vitality. Med Hypotheses 2006;66:832–43.
12 Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M. Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab 2007;6:280–93.
13 Ristow M, Zarse K. How increased oxidative stress promotes longevity and metabolic health: the concept of mitochondrial hormesis (mitohormesis). Exp Gerontol 2010;45:410–8.
14 Sharma PK, Agrawal V, Roy N. Mitochondria-mediated hormetic response in life span extension of calorie-restricted *Saccharomyces cerevisiae*. Age (Dordr) 2011;33:143–54.
15 Ristow M, Schmeisser S. Extending life span by increasing oxidative stress. Free Radic Biol Med 2011;51:327–36.
16 Ralser M, Benjamin IJ. Reductive stress on life span extension in *C. elegans.* BMC Res Notes 2008;1:19.
17 De Petrocellis L, Di Marzo V. Cell signalling: why fasting worms age slowly. Nature 2011;12(473):161–3.
18 Lee SJ, Hwang AB, Kenyon C. Inhibition of respiration extends *C. elegans* life span via reactive oxygen species that increase HIF-1 activity. Curr Biol 2010;20:2131–6.
19 Yang W, Hekimi S. A mitochondrial superoxide signal triggers increased longevity in *Caenorhabditis elegans*. PLoS Biol 2010; 8:e1000556.
20 Durieux J, Wolff S, Dillin A. The cell-non-autonomous nature of electron transport chain-mediated longevity. Cell 2011;144: 79–91.
21 Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 2006;443:787–95.
22 Miwa S, Riyahi K, Partridge L, Brand MD. Lack of correlation between mitochondrial reactive oxygen species production and life span in Drosophila. Ann NY Acad Sci 2004;1019:388–91.
23 Zheng J, Mutcherson R, II, Helfand SL. Calorie restriction delays lipid oxidative damage in *Drosophila melanogaster*. Aging Cell 2005;4:209–16.
24 Jacobson J, Lambert AJ, Portero-Otín M, Pamplona R, Magwere T, Miwa S, *et al.* Biomarkers of aging in Drosophila. Aging Cell 2010;9:466–77.
25 Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, *et al.* Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. Nature 2002;418: 344–8.
26 Tahara EB, Barros MH, Oliveira GA, Netto LE, Kowaltowski AJ. Dihydrolipoyl dehydrogenase as a source of reactive oxygen species inhibited by caloric restriction and involved in *Saccharomyces cerevisiae* aging. FASEB J 2007;21:274–83.
27 Guidi F, Magherini F, Gamberi T, Borro M, Simmaco M, Modesti A. Effect of different glucose concentrations on proteome of *Saccharomyces cerevisiae*. Biochim Biophys Acta 2010;1804:1516–25.
28 Agarwal S, Sharma S, Agrawal V, Roy N. Caloric restriction augments ROS defense in *S. cerevisiae*, by a Sir2p independent mechanism. Free Radic Res 2005;39:55–62.
29 Mesquita A, Weinberger M, Silva A, Sampaio-Marques B, Almeida B, Leão C, *et al.* Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H_2O_2 and superoxide dismutase activity. Proc Natl Acad Sci USA 2010;107:15123–38.
30 Barros MH, Bandy B, Tahara EB, Kowaltowski AJ. Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in *Saccharomyces cerevisiae*. J Biol Chem 2004;279:49883–8.
31 Magherini F, Carpentieri A, Amoresano A, Gamberi T, De Filippo C, Rizzetto L, *et al.* Different carbon sources affect lifespan and protein redox state during *Saccharomyces cerevisiae* chronological ageing. Cell Mol Life Sci 2009;66: 933–47.
32 da Cunha FM, Demasi M, Kowaltowski AJ. Aging and calorie restriction modulate yeast redox state, oxidized protein removal, and the ubiquitin-proteasome system. Free Radic Biol Med 2010;51:664–70.
33 Semchyshyn HM, Lozinska LM, Miedzobrodzki J, Lushchak VI. Fructose and glucose differentially affect aging and carbonyl/oxidative stress parameters in *Saccharomyces cerevisiae* cells. Carbohydr Res 2011;346:933–8.
34 Chipalkatti S, De AK, Aiyar AS. Effect of diet restriction on some biochemical parameters related to aging in mice. J Nutr 1983;113:944–50.
35 Koizumi A, Weindruch R, Walford RL. Influences of dietary restriction and age on liver enzyme activities and lipid peroxidation in mice. J Nutr 1987;117:361–7.
36 Laganiere S, Yu BP. Anti-lipoperoxidation action of food restriction. Biochem Biophys Res Commun 1987;145:1185–91.
37 Bevilacqua L, Ramsey JJ, Hagopian K, Weindruch R, Harper ME. Long-term caloric restriction increases UCP3 content but decreases proton leak and reactive oxygen species production in rat skeletal muscle mitochondria. Am J Physiol Endocrinol Metab 2005;289:E429–38.
38 Asami DK, McDonald RB, Hagopian K, Horwitz BA, Warman D, Hsiao A, *et al.* Effect of aging, caloric restriction, and uncoupling protein 3 (UCP3) on mitochondrial proton leak in mice. Exp Gerontol 2008;43:1069–76.
39 Ward WF, Qi W, Van Remmen H, Zackert WE, Roberts LJ, II, Richardson A. Effects of age and caloric restriction on lipid peroxidation: measurement of oxidative stress by F2-isoprostane levels. J Gerontol A Biol Sci Med Sci 2005;60:847–51.
40 Martini C, Pallottini V, De Marinis E, Marino M, Cavallini G, Donati A, *et al.* Omega-3 as well as caloric restriction prevent the age-related modifications of cholesterol metabolism. Mech Ageing Dev 2008;129:722–7.
41 Sohal RS, Agarwal S, Candas M, Forster MJ, Lal H. Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. Mech Ageing Dev 1994; 76:215–24.
42 Forster MJ, Sohal BH, Sohal RS. Reversible effects of long-term caloric restriction on protein oxidative damage. J Gerontol A Biol Sci Med Sci 2000;55:B522–9.
43 Dubey A, Forster MJ, Lal H, Sohal RS. Effect of age and caloric intake on protein oxidation in different brain regions and on behavioral functions of the mouse. Arch Biochem Biophys 1996;333:189–97.
44 Qiu X, Brown K, Hirschey MD, Verdin E, Chen D. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell Metab 2010;12:662–7.
45 Tian L, Cai Q, Bowen R, Wei H. Effects of caloric restriction on age-related oxidative modifications of macromolecules and lymphocyte proliferation in rats. Free Radic Biol Med 1995;19: 859–65.
46 Shinmura K, Tamaki K, Sano M, Nakashima-Kamimura N, Wolf AM, Amo T, *et al.* Caloric restriction primes mitochondria for ischemic stress by deacetylating specific mitochondrial proteins of the electron transport chain. Circ Res 2011;109: 396–406.
47 Chung MH, Kasai H, Nishimura S, Yu BP. Protection of DNA damage by dietary restriction. Free Radic Biol Med 1992;12: 523–5.
48 Kaneko T, Tahara S, Matsuo M. Retarding effect of dietary restriction on the accumulation of 8-hydroxy-2′-deoxyguanosine in organs of Fischer 344 rats during aging. Free Radic Biol Med 1997;23:76–81.
49 Loft S, Fischer-Nielsen A, Jeding IB, Vistisen K, Poulsen HE. 8-hydroxydeoxyguanosine as a urinary biomarker of oxidative DNA damage. J Toxicol Environ Health 1993;40:391–404.
50 Sanz A, Caro P, Ibañez J, Gómez J, Gredilla R, Barja G. Dietary restriction at old age lowers mitochondrial oxygen radical production and leak at complex I and oxidative DNA damage in rat brain. J Bioenerg Biomembr 2005;37:83–90.
51 Lass A, Sohal BH, Weindruch R, Forster MJ, Sohal RS. Caloric restriction prevents age-associated accrual of oxidative damage to mouse skeletal muscle mitochondria. Free Radic Biol Med 1998;25:1089–97.
52 Kang CM, Kristal BS, Yu BP. Age-related mitochondrial DNA deletions: effect of dietary restriction. Free Radic Biol Med 1998; 24:148–54.

53 Gredilla R, Sanz A, Lopez-Torres M, Barja G. Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart. FASEB J 2001;15:1589–91.

54 Gredilla R, Barja G, López-Torres M. Effect of short-term caloric restriction on H_2O_2 production and oxidative DNA damage in rat liver mitochondria and location of the free radical source. J Bioenerg Biomembr 2001;33:279–87.

55 Hagopian K, Harper ME, Ram JJ, Humble SJ, Weindruch R, Ramsey JJ. Long-term calorie restriction reduces proton leak and hydrogen peroxide production in liver mitochondria. Am J Physiol Endocrinol Metab 2005;288:E674–84.

56 Bevilacqua L, Seifert EL, Estey C, Gerrits MF, Harper ME. Absence of uncoupling protein-3 leads to greater activation of an adenine nucleotide translocase-mediated proton conductance in skeletal muscle mitochondria from calorie restricted mice. Biochim Biophys Acta 2010;1797:1389–97.

57 Faulks SC, Turner N, Else PL, Hulbert AJ. Calorie restriction in mice: effects on body composition, daily activity, metabolic rate, mitochondrial reactive oxygen species production, and membrane fatty acid composition. J Gerontol A Biol Sci Med Sci 2006;61:781–94.

58 Garait B, Couturier K, Servais S, Letexier D, Perrin D, Batandier C, *et al.* Fat intake reverses the beneficial effects of low caloric intake on skeletal muscle mitochondrial H_2O_2 production. Free Radic Biol Med 2005;39:1249–61.

59 Sanz A, Gredilla R, Pamplona R, Portero-Otín M, Vara E, Tresguerres JA, *et al.* Effect of insulin and growth hormone on rat heart and liver oxidative stress in control and caloric restricted animals. Biogerontology 2005;6:15–26.

60 Lambert AJ, Merry BJ. Effect of caloric restriction on mitochondrial reactive oxygen species production and bioenergetics: reversal by insulin. Am J Physiol Regul Integr Comp Physiol 2004;286:R71–9.

61 Lambert AJ, Wang B, Merry BJ. Exogenous insulin can reverse the effects of caloric restriction on mitochondria. Biochem Biophys Res Commun 2004;316:1196–201.

62 López-Torres M, Gredilla R, Sanz A, Barja G. Influence of aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria. Free Radic Biol Med 2002;32:882–9.

63 Ash CE, Merry BJ. The molecular basis by which dietary restricted feeding reduces mitochondrial reactive oxygen species generation. Mech Ageing Dev 2011;132:43–54.

64 Ketonen J, Pilvi T, Mervaala E. Caloric restriction reverses high-fat diet-induced endothelial dysfunction and vascular superoxide production in C57Bl/6 mice. Heart Vessels 2010;25:254–62.

65 Colom B, Oliver J, Roca P, Garcia-Palmer FJ. Caloric restriction and gender modulate cardiac muscle mitochondrial H_2O_2 production and oxidative damage. Cardiovasc Res 2007;74:456–65.

66 Seo AY, Hofer T, Sung B, Judge S, Chung HY, Leeuwenburgh C. Hepatic oxidative stress during aging: effects of 8% long-term calorie restriction and lifelong exercise. Antioxid Redox Signal 2006;8:529–38.

67 Cerqueira FM, da Cunha FM, Caldeira da Silva CC, Chausse B, Romano RL, Garcia CC, *et al.* Long-term intermittent feeding, but not caloric restriction, leads to redox imbalance, insulin receptor nitration, and glucose intolerance. Free Radic Biol Med 2011;51:1454–60.

68 Lambert AJ, Merry BJ. Lack of effect of caloric restriction on bioenergetics and reactive oxygen species production in intact rat hepatocytes. J Gerontol A Biol Sci Med Sci 2005;60:175–80.

69 Gredilla R, Phaneuf S, Selman C, Kendaiah S, Leeuwenburgh C, Barja G. Short-term caloric restriction and sites of oxygen radical generation in kidney and skeletal muscle mitochondria. Ann NY Acad Sci 2004;1019:333–42.

70 Ramsey JJ, Hagopian K, Kenny TM, Koomson EK, Bevilacqua L, Weindruch R, *et al.* Proton leak and hydrogen peroxide production in liver mitochondria from energy-restricted rats. Am J Physiol Endocrinol Metab 2004;286:E31–40.

71 Gredilla R, López-Torres M, Barja G. Effect of time of restriction on the decrease in mitochondrial H_2O_2 production and oxidative DNA damage in the heart of food-restricted rats. Microsc Res Tech 2002;59:273–7.

72 Cerqueira FM, Laurindo FR, Kowaltowski AJ. Mild mitochondrial uncoupling and calorie restriction increase fasting eNOS, akt and mitochondrial biogenesis. PLoS One 2011;6:e18433.

73 Sorensen M, Sanz A, Gómez J, Pamplona R, Portero-Otín M, Gredilla R, *et al.* Effects of fasting on oxidative stress in rat liver mitochondria. Free Radic Res 2006;40:339–47.

74 Laganiere S, Yu BP. Effect of chronic food restriction in aging rats II. Liver cytosolic antioxidants and related enzymes. Mech Ageing Dev 1989;48:221–30.

75 Rao G, Xia E, Nadakavukaren MJ, Richardson A. Effect of dietary restriction on the age-dependent changes in the expression of antioxidant enzymes in rat liver. J Nutr 1990;120:602–9.

76 Luhtala TA, Roecker EB, Pugh T, Feuers RJ, Weindruch R. Dietary restriction attenuates age-related increases in rat skeletal muscle antioxidant enzyme activities. J Gerontol 1994;49:B231–8.

77 De Cabo R, Cabello R, Rios M, López-Lluch G, Ingram DK, Lane MA, *et al.* Calorie restriction attenuates age-related alterations in the plasma membrane antioxidant system in rat liver. Exp Gerontol 2004;39:297–304.

78 Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, *et al.* Effects of caloric restriction on mitochondrial function and gene transcripts in rat muscle. Am J Physiol Endocrinol Metab 2002;283:E38–43.

79 Rohrbach S, Gruenler S, Teschner M, Holtz J. The thioredoxin system in aging muscle: key role of mitochondrial thioredoxin reductase in the protective effects of caloric restriction? Am J Physiol Regul Integr Comp Physiol 2006;291:R927–35.

80 Wardman P. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. Free Radic Biol Med 2007;43:995–1022.

81 Wardman P. Use of the dichlorofluorescein assay to measure 'reactive oxygen species'. Radiat Res 2008;170:406–7.

82 Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, *et al.* Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 2005;310:314–7.

83 Wrona M, Wardman P. Properties of the radical intermediate obtained on oxidation of 2′,7′-dichlorodihydrofluorescein, a probe for oxidative stress. Free Radic Biol Med 2006;41:657–67.

84 Oliveira GA, Tahara EB, Gombert AK, Barros MH, Kowaltowski AJ. Increased aerobic metabolism is essential for the beneficial effects of caloric restriction on yeast life span. J Bioenerg Biomembr 2008;40:381–8.

85 Scaduto RC, Jr, Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 1999;76:469–77.