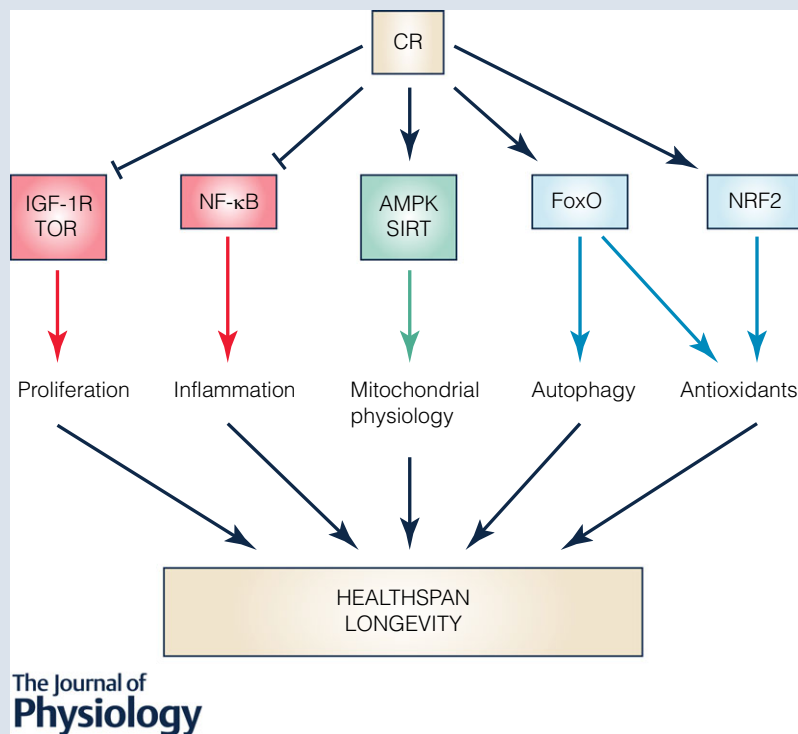


TOPICAL REVIEW

Calorie restriction as an intervention in ageing

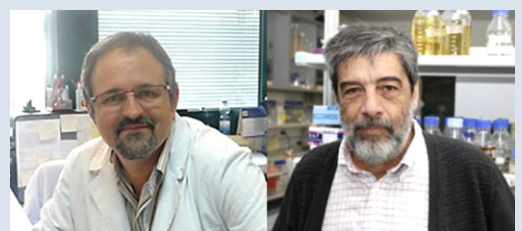
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Abstract Ageing causes loss of function in tissues and organs, is accompanied by a chronic inflammatory process and affects life- and healthspan. Calorie restriction (CR) is a non-genetic intervention that prevents age-associated diseases and extends longevity in most of the animal models studied so far. CR produces a pleiotropic effect and improves multiple metabolic pathways, generating benefits to the whole organism. Among the effects of CR, modulation of mitochondrial activity and a decrease in oxidative damage are two of the hallmarks. Oxidative damage is reduced by the induction of endogenous antioxidant systems and modulation of the peroxidability index in cell membranes. Mitochondrial activity changes are regulated by inhibition

Guillermo López-Lluch (left) and **Plácido Navas** (right) work at the Andalusian Centre for Development Biology (www.CABD.es), University of Pablo de Olavide in Sevilla, Spain. Their major research interest is the study of the regulation of bioenergetics pathways, mainly during ageing, and inherited mitochondrial disorders. Both approaches are based on the translational analysis of the coenzyme Q biosynthesis pathway in cell and mouse models. The biochemistry and molecular analysis of coenzyme Q in patients' fibroblasts are used for diagnosis of the mitochondrial disorder and the molecular mechanisms of the defect are studied in transgenic mouse models and dietary interventions in ageing mice.



of IGF-1 and Target of Rapamycin (TOR)-dependent activities and activation of AMP-dependent kinase (AMPK) and the sirtuin family of proteins. The activity of PGC-1 α and FoxO is regulated by these systems and is involved in mitochondria biogenesis, oxidative metabolism activity and mitochondrial turnover. The use of mimetics and the regulation of common factors have demonstrated that these molecular pathways are essential to explain the effect of CR in the organism. Finally, the anti-inflammatory effect of CR is an interesting emerging factor to be taken into consideration. In the present revision we focus on the general effect of CR and other mimetics in longevity, focusing especially on the cardiovascular system and skeletal muscle.

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Abstract figure legend Calorie restriction modifies several essential pathways in the organisms. Molecular components of this response act by modifying physiological processes such as proliferation, inflammation, mitochondrial physiology and remodelling, autophagy and the expression of antioxidants. In general CR inhibits processes involved in cell proliferation and glycolysis through blocking IGF-I receptor-dependent pathways and Target of Rapamycin (TOR). At the same time, CR induces mitochondrial activity by activating AMP-dependent kinase (AMPK) and sirtuins. This effect is accompanied by a higher activity of Forkhead box proteins (FoxO) that activate both auto/mitophagy and antioxidant expression. Interestingly, this FoxO activation can be induced by both, TOR inhibition and AMPK activation. Nuclear factor erythroid 2-related factor 2 (NRF2) is also activated by CR and, by this mechanism, the expression of antioxidant proteins is induced. Finally, CR also inhibits Nuclear Factor- κ B activity and reduces the proinflammatory profile found during ageing. Any of these processes contribute individually in the increase in healthspan and longevity found after CR.

Abbreviations AMPK, AMP-dependent kinase; ATG, autophagy-related; CR, calorie restriction; FoxO, Forkhead box proteins; NRF2, nuclear factor erythroid 2-related factor 2; PMRS, plasma membrane redox system; TOR, Target of Rapamycin.

Introduction

Ageing can be defined as a natural and progressive process occurring in all the living organisms that is characterized by the deterioration of structure and functional capacities. These alterations are multifactorial and involve diverse processes such as genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular communication (Lopez-Otin *et al.* 2013; Kennedy *et al.* 2014). Ageing is not a disease and therefore, disease-oriented research and treatment approaches are not adequate. It has thus been proposed that the use of health-oriented and preventive strategies is more beneficial than disease-oriented treatments (Rattan, 2014). As a process that affects the different organs, ageing is characterized for the accumulation of multiple chronic diseases caused by the same mechanisms that drive ageing. The study of this multimorbidity and the identification of molecular markers can help us to understand the biology of ageing and to develop strategies to delay it (Burkle *et al.* 2015; Fabbri *et al.* 2015).

Calorie restriction (CR) is, to date, the most successful intervention to delay ageing progression or the development of age-related chronic diseases. CR has been defined as the reduction of energy intake without

malnutrition. During the last few years it has been demonstrated that CR extends lifespan, extending the healthspan by delaying the onset of age-related diseases in many of the animal models studied (reviewed in Speakman & Mitchell, 2011). This effect of CR on longevity was explained in a unified theory of ageing proposed by David Sinclair (Sinclair, 2005). He considered that CR is not a simple and passive effect but an active, highly conserved stress response that increases the organism's chance of surviving adversity. Thus, CR produces a response that modifies key process in cell protection, reparation mechanisms and modulation of metabolism that permits a higher survival against adversity. This has been supported by the 'Hormesis hypothesis of CR' that suggests that the induction of a moderate stress causes adaptive responses of cells and organs, preventing further damage due to a stronger stress (Hepkiss, 2007; Masoro, 2007; Mattson, 2008; Rattan, 2008; Martins *et al.* 2011; Barbieri *et al.* 2013; Stankovic *et al.* 2013; Testa *et al.* 2014). As in CR, other factors such as exercise and some bioactive compounds found in fresh vegetables have been considered factors that induce hormetic responses in the organism affecting life and healthspan (Mattson, 2008).

In this review we will focus on the effect of CR and other mimetics on longevity and healthspan, focusing especially on cardiovascular system and skeletal muscle. Ageing is associated with a slow, but progressive muscle weakness,

which is largely attributable to muscle wasting. CR is able to prevent muscle damage (McKiernan *et al.* 2011) and reverse ageing cardiomyopathy (Bales & Kraus, 2013; Yan *et al.* 2013).

Calorie restriction and lifespan extension

Since the first reports of Osborne *et al.* and Loeb and Northrop in 1917 (Loeb & Northrop, 1917; Osborne *et al.* 1917) and the seminal report of McCay in 1935 (McCay *et al.* 1935), it is accepted that the rate of ageing can be affected by the amount of food consumed and hundreds of reports have confirmed the effect of CR on longevity, affecting most of the organisms tested to date. In these studies CR shows the capacity to extend longevity, affecting both, median and maximum lifespan in different organisms from yeasts to mammals (Testa *et al.* 2014). These species include *S. cerevisiae*, *C. elegans*, *D. melanogaster* and mammals such as *M. musculus* and *R. norvegicus* as model organisms. Other reports have shown effects on several other species such as rotifers, silkworms, spiders, fishes, dogs, etc. (Le Bourg, 2010). In primates, three separated studies of the effect of CR started at the 1980s using rhesus monkeys as a model (*Macaca mulatta*). These animals show many affinities to humans (Roth *et al.* 2004). Although the experiments with these animals are still ongoing, the results obtained to date are positive and, at least, an increase in healthspan has been found in CR-fed animals (Zainal *et al.* 2000; Roth *et al.* 2001; Mattison *et al.* 2012; Mercken *et al.* 2013; Colman *et al.* 2014). In humans, the most complete study on the effect of CR is the CALERIE (Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy) study, which started around seven years ago. The first reports have demonstrated similar CR-dependent effects as in mammalian models such as improving cardiovascular factors and insulin sensitivity (Pittas *et al.* 2005; Redman *et al.* 2014).

Although it is widely considered that the reduction in caloric intake itself is the main factor responsible for life extension, other studies have demonstrated that the reduction of one component in the diet such as methionine produces similar effects (Masoro, 2006). The main problem is the variability of diets and ingredients used in CR studies (Pugh *et al.* 1999). The importance of the dietary composition on retardation of ageing has been recently reinforced by two studies carried out in rhesus macaques where the discrepancies between the effect of CR on longevity seem to depend on the different diets used (Cava & Fontana, 2013; Colman *et al.* 2014). Interestingly, a new and controlled medium used for *Drosophila* feeding permits control of the specific nutrient that mimics the CR effect. Although effecting minimum changes in longevity, this medium permits determination of which nutrient is more effective in the response of the organism to CR (Piper

et al. 2005, 2014). Further, the use of other models of CR such as every-other-day feeding produces a lower intake of calories but induces changes similar to those found with classic CR (Rodriguez-Bies *et al.* 2010, 2015). On the other hand, recent studies performed on the macronutrients involved in CR indicate that the protein/carbohydrate ratio also plays an important role in the response of nutrient sensors (Solon-Biet *et al.* 2014). Thus, it seems that many factors influence the response of the organism to diets such as the number of calories, the quality of the source of calories and the induction of a nutritional stress by the every-other day feeding procedure which activates evolutionarily conserved molecular mechanisms involved in the CR effect on longevity (Goodrick *et al.* 1982; Anson *et al.* 2005).

There are no detailed reports about the effect of CR on longevity in humans. The longer life expectancy of humans in comparison with other animals and the low number of persons tested makes it difficult to reach conclusions about the effect of CR on human longevity (Robert, 2013). It is not yet clear if the reported effects on longevity and healthspan found in humans are due to the decrease in the calorie intake or are the result of a high quality diet (Rizza *et al.* 2014). However, it seems clear that a reduction in calorie intake in humans improves healthspan (Anderson & Weindruch, 2012), and delays cardiac ageing, improving cardiovascular function, one of the main causes of death in humans (Bales & Kraus, 2013).

Mitochondrial activity and ROS production are modulated by CR

In spite of the enormous number of articles published about the mechanism involved in the effect of CR on longevity, these mechanisms have not been clarified to date, although an important role of the maintenance of a balanced activity in mitochondria is supported by a large body of evidence. Interestingly, although differences are found in some pathways in the models used in the study of ageing and CR, the downregulation of genes participating in energy production in *Drosophila* and rodents indicates similar physiological effects during ageing (Augustin & Partridge, 2009).

Ageing is associated with the impairment of mitochondria, with a significant increase in ROS generation and a decrease in antioxidant defences, causing accumulation of mitochondrial DNA and oxidative damage (Merry, 2002; Chistiakov *et al.* 2014). In fact, the two parameters that have shown strong correlation with longevity are the increase in ROS production at mitochondria and other systems and the degree of fatty acid unsaturation in membranes (Barja, 2014). It is accepted that the longevity-promoting effects of CR can be explained, at least in part, by the modulation of mitochondrial and antioxidant activities in cells and

tissues (Lopez-Lluch *et al.* 2006, 2008). Furthermore, a recent review has concluded that mitochondria play a key role in the pathophysiology of ageing and in earlier stages of events leading to the ageing phenotype (Gonzalez-Freire *et al.* 2015). The mitochondrial electron transport chain is considered the main source of ROS in cells. Damaged mitochondria show reduced efficiency of energy production at the same time as higher levels of ROS release. It has been suggested that all the complexes of the mitochondrial electron transport chain are involved in ROS production in the resting state of muscle (Goncalves *et al.* 2015). Further, it is accepted that respiratory complexes assembled in supercomplexes regulate ROS production, and that a progressive deterioration of supercomplexes in ageing cause an irreversible increase in ROS (Genova & Lenaz, 2015). Thus, the maintenance of the structure of complexes is essential to avoid accumulation of ROS sources in mitochondria.

It has been suggested that ROS are the main source of mitochondrial DNA (mtDNA) mutations, affecting both point mutations and deletions that accumulate in different tissues during ageing in mammals, including humans (Trifunovic *et al.* 2004). These mutations are responsible for respiratory chain age-associated deficiencies in different tissues such as heart, skeletal muscle and brain. Thus, mice showing deficiency in mtDNA polymerase develop a mtDNA mutator phenotype, including higher levels of point mutations and deleted mtDNA that have been associated with the premature onset of ageing and ageing-related phenotypes (Trifunovic *et al.* 2004).

Mitochondrial malfunction can also affect the whole physiology of cells. For example, an increase in ROS in skeletal muscle has been associated with insulin resistance in obese individuals, appearance of type 2 diabetes and muscle malfunction during normal ageing (Barbieri *et al.* 2013), indicating that a rise in ROS levels can lead to deterioration of the physiology of the whole cell, tissue and organ.

It has been demonstrated that CR lowers mitochondrial membrane potential and consequently the production of ROS at the same time that it modifies the saturation/unsaturation index in mitochondrial membranes preventing oxidative damage and maintaining membrane fluidity. Recently, we have demonstrated that the production of H₂O₂ linked to complexes I and III is reduced by CR in both muscle (Chen *et al.* 2012, 2014) and liver (Chen *et al.* 2013). This effect was found in young animals after just 1 month of CR (Chen *et al.* 2012) and was maintained after 8 months of CR (Chen *et al.* 2014).

Another important factor involved in the accumulation of damaged mitochondria during ageing is the decline of the mitochondrial turnover by inhibition of mitophagy; the specific autophagy process that removes damaged mitochondria (Chistiakov *et al.* 2014). It is clear that the renovation of mitochondrial network plays a key role in

healthspan increase after CR (Lopez-Lluch *et al.* 2008). Further, the correct functioning of the quality control mechanism of mitochondria has been suggested as a crucial factor in counteracting the ageing process (Weber & Reichert, 2010).

Besides these strong lines of evidence, the age of the organisms at the onset of CR seems to play an important role in the regulation of mitochondrial activity. In rhesus monkey studies, ageing resulted in up-regulation of transcripts affecting inflammation and oxidative stress and down-regulation of genes involved in mitochondrial electron transport chain and oxidative phosphorylation (Kayo *et al.* 2001). These authors did not observe beneficial effects of adult-onset CR at the transcriptional level (Kayo *et al.* 2001), although the same animal cohort showed several physiological beneficial effects of CR such as higher insulin sensitivity (Kemnitz *et al.* 1994) and decreased production of pro-inflammatory cytokines by peripheral blood mononuclear cells (Kim *et al.* 1997). Interestingly, in previous studies, more of the 80% of age-related changes in gene expression found in skeletal muscle were partially or completely suppressed by early onset of CR in mice (Lee *et al.* 1999). These different results probably indicate an age-dependent effect of CR, as we have found in murine models (Rodriguez-Bies *et al.* 2015; Tung *et al.* 2015b).

Importance of membrane lipid composition on the CR effect

As indicated above, the decrease in the oxidative damage in organic structures is one of the main factors contributing to lifespan extension induced by CR (Sohal & Weindruch, 1996; Gredilla & Barja, 2005). The fatty acid composition of cell membranes is another important factor involved in ageing progression because it influences the lipid peroxidation rate during ageing (Hulbert, 2005). Thus, several findings indicate that the increase in lipid peroxidation during ageing (Yu, 2005), which is due to different factors such as enrichment in more oxidizable polyunsaturated fatty acids (e.g. 22:6), reduction of membrane-linked antioxidant activities and higher production of ROS, affects the main processes in cells from plasma membrane activities to mitochondrial function.

It is still unclear whether lifespan extension induced by CR can also be explained by changes in membrane fatty acid composition conferring higher resistance to peroxidation. We have recently found that lipid composition in the diet can modulate the effect of CR on longevity (Lopez-Dominguez *et al.* 2015). One month of CR produced minor changes in fatty acid composition of muscle mitochondrial phospholipids, mainly affecting phosphatidyl-choline (Chen *et al.* 2012) and increasing monounsaturated fatty acid (MUFA) levels in liver mitochondria (Chen *et al.* 2013). These small

changes in membrane lipid composition have been related to other changes such as mitochondrial proton leak, mitochondrial electron transport chain activities, ROS generation and lipid peroxidation (Chen *et al.* 2012, 2013) and also mitochondrial fission/fusion dynamics and apoptotic signalling (Khraiwesh *et al.* 2013, 2014; Lopez-Dominguez *et al.* 2013).

Antioxidant activities in ageing and CR

Oxidative damage is prevented by endogenous antioxidant activities in cells and organs. A battery of enzymes are involved in the elimination of ROS such as superoxide (mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD) and hydroperoxides (catalase and glutathione peroxidase), with the glutathione-dependent system being the most important in cells (Lenaz *et al.* 1999). Although one of the most popular theories to explain the longevity effect of CR on different organisms is based on higher protection against the increase in oxidative stress and subsequent cell damage, the role of antioxidants in CR effect is not clear. Many lines of evidence indicate that CR reduces age-associated accumulation of oxidized molecules (Gredilla & Barja, 2005). However, the lack of lifespan extension in antioxidant enzyme overexpression experiments casts doubt on the importance of antioxidants in the CR effect (Muller *et al.* 2007). Higher levels of antioxidants do not necessarily indicate a higher antioxidant protection and imbalances produced by the overexpression or higher activity of one antioxidant enzyme must be taken into consideration. Besides the important number of studies that indicate a modulation of antioxidant activities in cells and organs by CR, this effect seems to be organ dependent and studies designed

to clarify how ageing and CR affect antioxidant enzymes in each individual tissue and organ must be performed (Ji *et al.* 1990; Tung *et al.* 2015b).

Importantly, oxidoreductases such as Cyt_B₅Rase and NAD(P)H:quinone oxidoreductase 1 (NQO1) are involved in the prevention of lipid peroxidation through a coenzyme Q-dependent system present in cell membranes and especially in plasma membrane. This system is known as the plasma membrane redox system (PMRS) and is also involved in the maintenance of the redox cycle of vitamin E (Fig. 1 Navas *et al.* 2005, 2007). Several years ago, we demonstrated that CR induces redox coenzyme Q-dependent activities at the plasma membrane in old mouse and rat liver but not in young animals (De Cabo *et al.* 2004; Lopez-Lluch *et al.* 2005). This age-dependent effect was further confirmed in brain (Hyun *et al.* 2006) and in muscle (Rodriguez-Bies *et al.* 2015). Therefore, the age of the organisms seems to play an important role in the relationship of CR with membrane-linked antioxidant activities.

The activity of the PMRS is also involved in the maintenance of mitochondrial homeostasis. In a recent work, it has been demonstrated that overexpression of NQO1 is associated with higher neuroprotection against energetic and proteotoxic stress but not against oxidative stress, indicating the importance of this system in the maintenance of bioenergetics and a secondary role in oxidative prevention (Hyun *et al.* 2012). Furthermore, NQO1 up-regulates mitochondrial function in human neuroblastoma cells (Kim *et al.* 2013). Experiments performed in *S. cerevisiae* have demonstrated that the activation of NQR1, an orthologue of NQO1, is associated with the transition from fermentation to mitochondrial-dependent oxidative

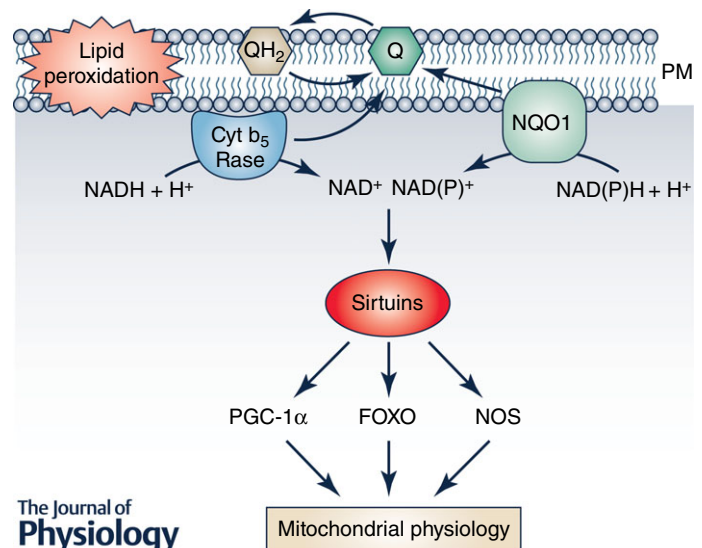


Figure 1. Activities of the plasma membrane redox system

The Q-dependent enzymatic activities at the plasma membrane (PM) not only prevent oxidative damage affecting lipid peroxidation through reduced Q or by maintaining vitamin E turnover in the membrane, but can also show other regulatory activities in the cytosol. The oxidation of NADH or NAD(P)H in the PM environment can regulate the activity of NAD⁺-dependent deacetylases such as sirtuins, mainly SIRT1. These sirtuins will further regulate the activity of many other proteins involved in the control of metabolism and mitochondrial turnover such as PGC-1α, FOXO or NOS among others. These proteins are involved in the regulation of mitochondrial biogenesis, turnover and oxidative activity. Thus, upregulation of PMRS by CR in old animals can be linked to a higher SIRT1 activity and the regulation of mitochondrial function in old animals.

metabolism (Jimenez-Hidalgo *et al.* 2009). Further, Sqstm1/p62, a protein that mediates nuclear factor erythroid 2-related factor 2 (NRF2) activation and increases NQO1, is essential in the maintenance of mitochondrial membrane activity during ageing in mice (Kwon *et al.* 2012), indicating the importance of this member of the coenzyme Q-dependent oxidoreductases in maintaining mitochondrial integrity and longevity. It seems clear that the induction of the NRF2/ARE pathway during CR not only increases antioxidant protection in membranes by activating PMRS but also is essential for control of metabolic homeostasis during ageing (Ungvari *et al.* 2008).

Interestingly, recent studies performed in humans demonstrated that resveratrol, a compound able to induce many of the CR-triggered pathways, up-regulates human erythrocyte PMRS, mitigating the alterations induced by oxidation during ageing (Pandey & Rizvi, 2013). The same authors have suggested the direct relationship of this activity with the higher lifespan found in long-living organisms (Rizvi *et al.* 2011). However, the mechanisms involved in the prolongevity effect of PMRS activation and its importance in ageing are not clear and the difficulties in the determination of PMRS activity in some tissues obscures its role in ageing and the effect of CR.

Taking into consideration the deleterious effect of lipid peroxidation on membrane function, can the higher activity of PMRS be related to membrane-linked cell signalling processes? Is it related to a higher sensitivity to local factors or hormones? Some evidence indicates that activation of the PMRS system and the prevention of the oxidation of membranes can be involved in the maintenance of the cell response to external stimuli. In fact, deletion of NRF2 impairs glucose tolerance in diabetic mice (Aleksunes *et al.* 2010) and NQO1 has been associated with both the maintenance of the metabolic machinery in pancreatic β -cells (Gray *et al.* 2011) and the prevention of its destruction induced by different stressors (Yeo *et al.* 2013). It has also been suggested that the regulation of this system can modulate the activity of different proteins at the plasma membrane and the nutrient-sensors sirtuins, importantly related to many CR effects in organisms (Crane & Low, 2005) and in the control of cell survival by apoptosis (Villalba & Navas, 2000).

Molecular mechanisms involved in the effect of CR on longevity

A decrease in the uptake of nutrients produces an imbalance in the metabolic system. To return to a new equilibrium, several regulatory pathways interact. The new equilibrium responds to the relationship between growth-associated pathways, such as the insulin–insulin growth factor-1 (IGF-I) receptor system and Target of

Rapamycin (TOR), and regulators of a more efficient respiratory metabolism such as AMP-dependent kinase (AMPK) and sirtuins, the NAD⁺-dependent deacetylases. Interestingly, the relationship between these mechanisms is conserved during evolution from yeasts to humans. In simple terms, the decrease in calories in the diet activates systems involved in a more efficient metabolism, a higher protection against cellular damage and the activation of remodelling mechanisms, whereas less efficient metabolism and synthetic pathways are blocked. Interestingly, these mechanisms regulate each other in all the organisms studied, indicating that CR activates an evolutionarily conserved response that avoids fruitless energy expenditure and use recycled structures for the organisms' survival (Fig. 2). In this section we provide a resumé of the main molecular mechanisms involved in the CR effect on lifespan and healthspan (more detailed information can be found in Michan, 2014; Testa *et al.* 2014).

IGF-I. Among the hypotheses to explain ageing, several findings indicate that changes in the insulin–IGF-I receptor signalling system are involved in the modulation of ageing in both, invertebrates (Tatar *et al.* 2003; Kenyon, 2011) and vertebrates (Dupont & Holzenberger, 2003; Selman *et al.* 2008). CR reduces plasma levels of IGF-I, insulin and glucose in rodents (Argentino *et al.* 2005) and also in humans (Weiss *et al.* 2006). Studies performed in *C. elegans* have shown that decrease in the activity of Daf-2 (similar to IGF-I receptor in these organisms) doubles lifespan (Kenyon, 2010). Activation of the IGF-I-dependent signalling cascade induces protein synthesis by activating TOR and ribosomal protein 6 kinase (S6K) and also inhibits the transcriptional activity of the forkhead box transcription factors (FoxO). These are important regulatory elements in the effect of CR on lifespan since the inhibition of the FoxO orthologue *daf-16* in both, *C. elegans* and *D. melanogaster* by IGF-I-dependent signalling decreases lifespan in these organisms (Kenyon, 2010). In humans, a genetic variation of the *Fox3A* gene has been found in long-lived and healthy men showing benefits in cardiovascular disease and insulin sensitivity (Willcox *et al.* 2008).

Interestingly, FoxO is involved in the induction of several stress response genes (Goto & Takano, 2009), indicating that this is an important mediator of the induction of protective mechanisms in the organisms after CR. This agrees with the 'Hormesis hypothesis' explaining the CR effect on ageing based on the effect that a moderate stress produces by induction of protective responses in the organisms (Masoro, 2007; Rattan, 2008).

Target of Rapamycin (TOR). TOR protein members are a conserved family of kinases that respond to stress, nutrient

and growth factors. TOR stimulates cell growth when food is available. TOR inhibits autophagy and stimulates protein synthesis and cell proliferation (Wullschleger *et al.* 2006). The importance of TOR in longevity induced by CR was also demonstrated in invertebrates such as *C. elegans* and *D. melanogaster*. In these organisms, down-regulation of TOR produces an increase in lifespan (Sharp, 2011). This same effect has been also found in mice (Harrison *et al.* 2009; Selman *et al.* 2009). Interestingly, a decrease in S6K activity, a protein kinase involved in protein synthesis and activated by TOR in yeast, worms, flies and mice, also

extends lifespan and delays the progression of age-related diseases (Selman *et al.* 2009). At the same time, the increase in FoxO-dependent autophagy found when TOR is inhibited is essential for lifespan extension in yeast (Kamada *et al.* 2004) and in *C. elegans* (Toth *et al.* 2008).

AMP-dependent protein kinase (AMPK). AMPK is a very sensitive energy sensor in cells and organisms. AMPK is activated in response to an increase in the AMP/ATP ratio, for example, when cells are deprived of glucose, whereas its activity decreases when cells are full of energy, indicated by a lower AMP/ATP ratio (Hardie, 2011). As in the case of other regulators such as sirtuins, its effect on longevity has been observed in several organisms from yeast to mammals. It has been clearly demonstrated that an increase in AMPK activity is associated with a longer lifespan while its inhibition shortens it (Apfeld *et al.* 2004; Harkness *et al.* 2004; Tohyama & Yamaguchi, 2010). However, in mammals, the importance of this kinase is under debate since it has been reported that its activity is not affected by CR (Gonzalez *et al.* 2004) or is even reduced (To *et al.* 2007). However, other studies indicate an increase in AMPK activity in heart and skeletal muscle (Jager *et al.* 2007; Miller *et al.* 2012). These discrepancies could be due to differences in the amount of time under CR or the degree of CR which can play an important role in nutrient balance. Further, as has been indicated in antioxidant responses (Rodriguez-Bies *et al.* 2015; Tung *et al.* 2015b), the age of the organism can be an important factor in the importance of each component that responds to CR.

Sirtuins. One of the best known effectors of CR is the family of NAD⁺-dependent protein deacetylases. They are found in all the organisms, from prokaryotes to humans, affecting the activity of many enzymes by removing acetyl residues. Some time ago it was demonstrated that the orthologue of mammalian SIRT-1, Sir2, was able to increase lifespan in *S. cerevisiae* and in invertebrates such as *C. elegans* and *D. melanogaster* (Kaerberlein *et al.* 1999; Tissenbaum & Guarente, 2001; Wood *et al.* 2004). In mammals, it is clear that CR induces the expression and the activity of sirtuins in many organs and their activities are associated with many of the metabolic effects found in these organisms after CR (Imai & Guarente, 2010).

Interestingly, sirtuins seem to play a central role in the response to CR. Sirtuins act as nutrient and metabolic sensors by detecting fluctuations in the NAD⁺/NADH ratio. When nutrients, especially glucose, decrease, NAD⁺ accumulates and sirtuins are activated. Thus sirtuins have an opposite effect to TOR activation after glucose input. The increasing number of proteins regulated by deacetylation, involved in several key aspects of cell physiology from the cell cycle to metabolism and antioxidant protection, highlights the importance of sirtuins in the

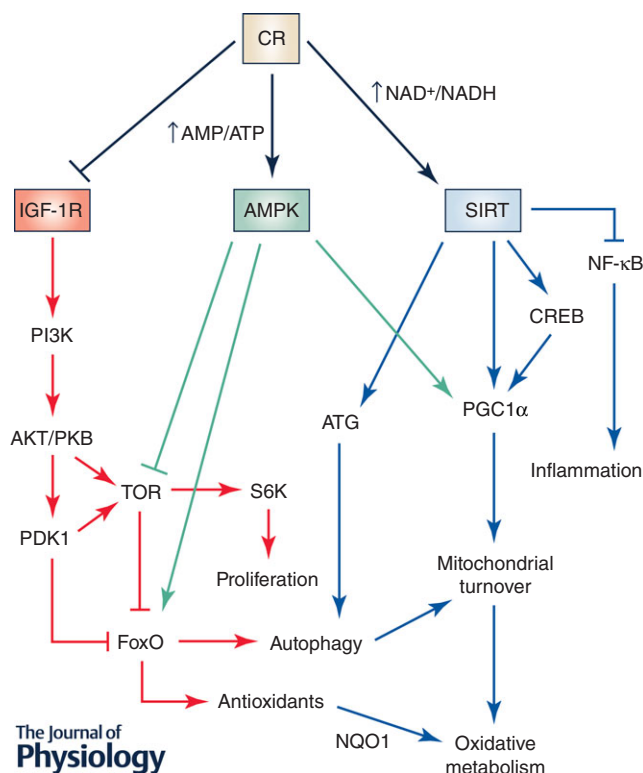


Figure 2. Complex interaction of proliferative and protective mechanisms in the CR longevity effect in organisms

Nearly all the organisms studied to date have shown similar mechanisms in response to CR. Basically, CR induces mechanisms involved in energy efficacy and inhibits mechanisms involved in less efficient energy consumption and proliferation. Thus, CR inhibits IGF-I-dependent signalling which activates TOR and protein synthesis and inhibits FoxO, blocking antioxidant expression and autophagy. On the other hand, the balance between AMP and ATP and NAD⁺ and NADH serves as a signal to activate AMPK and sirtuins, respectively. These nutrient sensors and regulators block the signal dependent on IGF-I and TOR at the same time as they activate mechanisms to enhance more efficient energy production through oxidative metabolism and mechanisms to enhance cell protection through antioxidants and organelle turnover through autophagy. At the same time, CR, through sirtuins, can block inflammation mediators. This regulation occurs at different levels and many mediators can produce a redundant response such as autophagy activation by blocking TOR-dependent FoxO inhibition or by inducing the activity of autophagy-related (ATG) proteins.

regulation of metabolism, insulin response, cell cycle, autophagy, etc. In fact, the role of sirtuins in the effect of CR on longevity and the increase in healthspan has been extensively reviewed (Guarente, 2011, 2013). However, sirtuin-independent mechanisms have been also proposed to explain the increase in longevity in yeast induced by CR (Kaeberlein *et al.* 2004). Also, the complexity of sirtuins in mammals has promoted the idea that they can show both pro- and anti-ageing capacities in mice (Kaeberlein, 2008; Li *et al.* 2008).

Mitochondrial modifications induced by CR

Taking into consideration the role of the factors induced by CR in metabolic regulation, their modulation can improve cellular physiology and maintain an organism's capacity for extended ageing. Several studies found that mitochondrial biogenesis is impaired during ageing, especially in high-energy-demanding tissues such as muscle, brain or heart (Conley *et al.* 2000; Short *et al.* 2005). CR and other interventions such as exercise or nutraceuticals such as resveratrol induce mitochondrial biogenesis in heart and skeletal muscle in humans and other organisms (Feige *et al.* 2008; Baur *et al.* 2010; Rodriguez-Bies *et al.* 2015), indicating their role in the maintenance of the mitochondrial activity in these organs during ageing.

At least three different pathways have been associated with this effect on mitochondrial biogenesis in muscle: eNOS induction, PGC-1 α activation and adiponectin-dependent activation of the SIRT1/AMPK axis. Sirtuins seem to play a key regulatory role in these pathways since SIRT1 knockout (KO) in skeletal muscle blunts the physiological changes induced by CR in this tissue (Schenk *et al.* 2011).

Nitric oxide synthase is induced by CR in mice and human muscle (Nisoli *et al.* 2005; Civitaresse *et al.* 2007) and plays an important role in CR-induced mitochondrial biogenesis, since in eNOS^{-/-} mice, the effect of CR is blocked (Nisoli *et al.* 2005). SIRT1 has also been suggested to play an essential role, since this enzyme activates eNOS by deacetylation (Mattagajasingh *et al.* 2007).

PGC-1 α is the main factor involved in the regulation of mitochondrial mass and the adaptation to energy demand. PGC-1 α activity is related to many of the beneficial effects of physical activity in model organisms (Li *et al.* 2011). PGC-1 α also stimulates mitochondrial biogenesis, regulates mitochondrial dynamics, modulates oxidative phosphorylation and controls mitochondrial genome copy number (Handschin & Spiegelman, 2006; Gouspillou *et al.* 2014). AMPK and SIRT1, the two main metabolic sensors in cells, directly modulate PGC-1 α activity through phosphorylation and deacetylation (Nemoto *et al.* 2005; Jager *et al.* 2007). In fact, it has been proposed that AMPK and SIRT1 are the most important

effectors that activate mitochondrial biogenesis through increasing PGC-1 α activity at the transcriptional and post-translational level (Martin-Montalvo & de Cabo, 2013). Recently, it has been demonstrated that both skeletal muscle-specific AMPK deficiency and AMPK α 2 KO mice impair the beneficial effects of CR on glucose tolerance due to reduced SIRT1 levels. Thus, considerable evidence indicates that the activity of AMPK and SIRT1 are essential for physiological responses induced by CR in muscle.

Among the other members of the sirtuin family, SIRT3 is located within mitochondria, suggesting its immediate regulation of mitochondrial activity. In fact, SIRT3 increases in skeletal muscle after CR and decreases on feeding a high-fat diet, indicating that regulation depends on the calorie intake (Palacios *et al.* 2009). This sirtuin has been linked to the induction of the catabolism of lipids (Kendrick *et al.* 2011). Among other important roles of SIRT3 in mitochondria, we can highlight the regulation of succinate-dehydrogenase activity by deacetylation (Kendrick *et al.* 2011), the modulation of the activity of Mn-SOD (Qiu *et al.* 2010) or the autophagy regulation by deacetylation of FoxO3 in CR-fed animals (Kume *et al.* 2010). Interestingly, SIRT3 KO mice show PGC-1 α down-regulation and low levels of AMPK phosphorylation after CR, indicating a key role for this mitochondrial sirtuin in energy regulation in muscle (Palacios *et al.* 2009). On the other hand, PGC-1 α exerts a positive feedback, stimulating SIRT3 expression, in muscle and liver (Kong *et al.* 2010).

Interestingly, the role of SIRT3 in acetate metabolism has been also related to ageing (Shimazu *et al.* 2010). Acetate plays an important role in cell metabolism, being an important product of ethanol and fatty acid metabolism, especially during fasting or starvation (Seufert *et al.* 1974). Acetate can be converted into acetyl-CoA by the activity of acetyl-CoA synthase enzymes in cytosol (AceCS1) or mitochondria (AceCS2). These enzymes are activated by deacetylation by both SIRT1 in cytosol and SIRT3 in mitochondria (Shimazu *et al.* 2010). An important role for SIRT3 in acetate metabolism has been suggested, since both, SIRT3 KO and AceCS2 KO mice show overlapping phenotypes. However, to date, no clear data about the role of acetate in the ageing progress has been shown although its AceCS-mediated synthesis in yeast has been associated with higher longevity (Falcon *et al.* 2010).

Another interesting factor involved in mitochondrial activity regulation after CR is the hormetic response linked to mitochondrial activity, called mitohormesis. Apart of the above-indicated factors that regulate mitochondrial biogenesis, a role for ROS production in mitohormesis has been proposed (Ristow & Zarse, 2010; Ristow & Schmeisser, 2011, 2014; Ristow, 2014). It has been proposed that higher ROS production after

CR induces the antioxidant response, increases pathways and molecules involved in removing cell damage and modulates mitochondrial activity (Ristow, 2014). Interestingly, it has been demonstrated that ROS are involved in the modulation of glucose metabolism and the increase in oxidative metabolism and stress resistance in CR and also in exercise (Barbieri *et al.* 2013). However, the relationship of ROS with mitohormesis and the regulation of enzymatic regulators such as AMPK, sirtuins or FoxO is still controversial, although an AMPK-dependent increase in SIRT3 activity has been associated with the activation of protection against oxidative stress (Brandauer *et al.* 2015) and the ROS-dependent activation of AMPK has recently been associated with higher longevity in *C. elegans* (Hwang *et al.* 2014) and with the increase in autophagy in HUVEC cells (Li *et al.* 2015). Therefore, it seems that mild oxidative stress could be associated with the response to CR. Although the mechanism involved in the ROS-induced increase in mitochondrial metabolism is not clear, it seems that AMPK-dependent modulation of FoxO or PGC-1 α could be involved. But more importantly, these findings indicate that the abuse of exogenous antioxidants during ageing may reduce lifespan rather than increasing it. It is more important to induce endogenous antioxidant systems that add antioxidants to compromised organisms lacking enzymes able to use them.

Further research is needed to clarify the complex network of interactions and protein modifications involved in metabolism regulation induced by CR and related to a higher energetic efficiency. However, it is clear that regulation of sirtuins activities are, together with AMPK activity and PGC-1 α -dependent pathways, at the centre of the response to CR (Fig. 3).

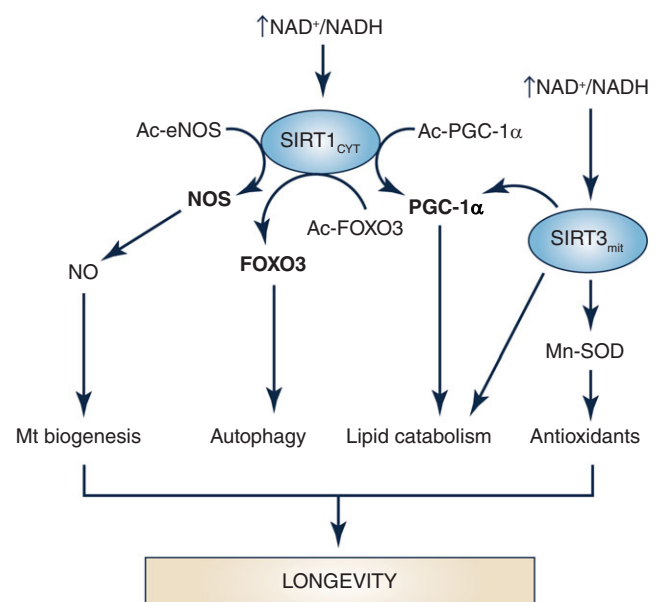
CR mimetics

During the past few years, a group of molecularly unrelated compounds have emerged as CR mimetics, able to produce, at least partially, similar effects in different organisms. In general, all these compounds have a common denominator, the activation of the above-described molecular pathways involved in the response to CR such as AMPK and sirtuins (Fig. 2).

Among these compounds, polyphenols have been extensively studied. These compounds are characterized by the presence of one or more hydroxyl groups bound to an unsaturated aromatic hydrocarbon ring such as benzene and are produced by plants in response to stress or fungal infections (Bravo, 1998). Among these compounds, resveratrol has emerged as the compound that best mimics the CR effect on several organisms (Testa *et al.* 2014). Among the demonstrated effects of resveratrol we can highlight: a decrease in insulin secretion and insulin level, an increase in insulin sensitivity, a decrease in fat mass, an increase in mitochondrial biogenesis and oxidative

phosphorylation, an increase in the NAD⁺/NADH ratio and the subsequent activation of sirtuins, an increase in AMPK activity and increase in mitophagy and autophagy (Testa *et al.* 2014). Interestingly, a recent study of humans demonstrates that treatment with resveratrol produces similar effects to CR in obese people. Thirty days of resveratrol treatment activated AMPK, and increased SIRT1 and PGC-1 α , improving muscle mitochondrial respiration based on fatty acids similarly to the effect found with CR (Timmers *et al.* 2011).

Another interesting compound is rapamycin (sirolimus), an inhibitor of TOR that induces significant lifespan extension in several organisms (Selman *et al.* 2009; Sharp, 2011). This compound has been shown to increase lifespan in yeast, nematodes, flies and human cells by decreasing protein synthesis, through inhibition of S6K, and by inducing autophagy through the inhibition of TOR and the subsequent activation of FoxO. However, in a recent study performed in mice, rapamycin has



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Figure 3. Role of sirtuins in the control of mitochondrial physiology

The increase in the NAD⁺/NADH ratio produced by CR in cells may induce the activity of the cytosolic SIRT1 and the mitochondrial SIRT3 forms. Both have been involved in the induction of many processes involved in the modulation of mitochondrial physiology such as induction of mitochondrial biogenesis by activating NOS and PGC-1 α , which, coupled with the activation of autophagy by deacetylation of FOXO3, promotes the renovation of mitochondria, eliminating damaged and unbalanced mitochondria at the same time as undamaged and active mitochondria increase. At the same time, lipid catabolism is promoted and the activity of mitochondrial antioxidants increases through the activity of SIRT3. Thus, sirtuins seem to be at the centre of mitochondrial modulation during CR.

shown a selective effect on female mice and different features in comparison with CR (Miller *et al.* 2014). Thus, its use as mimetic of CR needs more study, at least, in mammals.

In 1998, Lane and colleagues proposed that feeding animals with glycolysis blockers such as 2-deoxy-D-glucose (2DG) could mimic the physiological effects of CR (Lane *et al.* 1998). This compound increases *C. elegans* lifespan (Schulz *et al.* 2007) and produces several CR-related effects in rats such as a decrease in insulin levels (Ingram & Roth, 2011), an increase in glucocorticoids (Wan *et al.* 2004) and an increase in lifespan (Wan *et al.* 2003). As in the other cases, it seems that 2DG can also activate AMPK and SIRT1 (Wang *et al.* 2011; Yang *et al.* 2011). However, the main problem with this compound is that it can be cardiotoxic and increases the incidence of tumours in adrenal medulla in long-term treatments (Minor *et al.* 2010).

Another CR mimetic of interest is metformin, a biguanine drug, clinically used in the treatment of type-2 diabetes that has increased the lifespan of *C. elegans* and *D. melanogaster*, probably by activating AMPK (Slack *et al.* 2012). Experiments performed in mice indicate that low doses of metformin produce similar effects to those found with CR, such as higher insulin sensitivity, reduction of low-density lipoproteins and cholesterol levels in plasma, an increase in antioxidant protection and reduction of chronic inflammation. As in the above-indicated cases, the effect of metformin seems to be through activation of AMPK (Martin-Montalvo *et al.* 2013). Furthermore, the effect of metformin has recently been associated with the activation of SIRT1-mediated autophagy in an AMPK-independent pathway (Song *et al.* 2015). This is therefore a promising compound, since, in *in vitro* experiments with human fibroblasts, metformin has shown the capacity to restore the mitochondrial dysfunction found in aged cells (Alcocer-Gomez *et al.* 2015).

All these compounds have shown, at least in part, similar effects to CR on cells, tissues and organs and all of them have produced mitochondrial regulation by increasing turnover and activating oxidative metabolism through activation of the AMPK/SIRT1 axis and inhibition of TOR. It seems clear then that the regulation of mitochondrial metabolism by these nutrient sensors is at the centre of the effect of CR and its mimetics on healthspan and longevity.

CR and inflammation

Inflammation is also an important factor in ageing. Proinflammatory factors such as TNF- α , IFN- γ , IL-1 β , IL-18 and others increase systemically during ageing (Chung *et al.* 2002; Salvioli *et al.* 2006; Goto, 2008). It has been shown that the increase in oxidative stress during ageing can be involved in the incidence of age-related

diseases and the induction of a chronic inflammatory process (Solana *et al.* 2006; Candore *et al.* 2010). Evidence indicates that sterile inflammation, a chronic low-level inflammation, is caused by either stress or environmental conditions and increases during ageing (Feldman *et al.* 2015). Accumulation of damage during ageing increases the production of danger-associated molecular patterns (DAMPs) that are recognized by immune receptors and activates the inflammasome (Feldman *et al.* 2015). In fact, chronic inflammatory status has been associated with many chronic diseases of ageing (Pawelec *et al.* 2014) and 'inflammaging' has been coined as a new concept for chronic inflammation associated with ageing (Franceschi & Campisi, 2014).

The progressive muscle weakness linked to ageing has been associated with a diminished function of satellite cells. The activity of satellite cells can be affected by systemic inflammatory factors such as TNF- α that negatively affect muscle-regenerating capacity (Degens, 2010). In a recent study, it has been demonstrated that the chronic-inflammation-related acute phase C-reactive protein (CRP) negatively affects skeletal muscle mass in elderly women and serum from these elderly women reduces myoblasts growth (Wahlin-Larsson *et al.* 2014).

It is likely that the maintenance of mitochondria biogenesis by CR can increase the resistance of muscle against inflammation. The decrease in PGC-1 α protein during ageing has been associated with, among other factors, inflammatory markers in white adipose and liver tissues (Sczelecki *et al.* 2014). Furthermore, exercise prevents the age-associated increase in systemic IL-6 and TNF- α in a PGC1- α -dependent mechanism in mice (Olesen *et al.* 2013). CR also decreases inflammation and insulin resistance in an age-associated inflammation rat model (Horrillo *et al.* 2011) and shows anti-inflammatory activity by modulating GSH redox status, NF- κ B, SIRT1, PPARs, and FoxOs (Chung *et al.* 2011). And, recently we have also found that resveratrol is able to decrease both the production of proinflammatory cytokines and the induction of inflammasome in aged mice liver (Tung *et al.* 2015a).

Effect of CR on age-associated diseases in humans

Two of the main age-associated diseases in humans are type 2 diabetes and cardiovascular disease (CVD). In both cases, models of CR, dietary interventions or exercise have shown important improvements in the onset and development of these diseases. The onset of insulin resistance and type 2 diabetes, probably due to the impairment of fatty acid oxidation by mitochondrial dysfunction, is a hallmark of ageing (Civitaresse *et al.* 2006; Rolo & Palmeira, 2006). Interestingly, PGC-1 α expression and mitochondrial biogenesis stimulated by AMPK and SIRT1 in muscle has been associated with the reduction of insulin

resistance (Reznick & Shulman, 2006; Gerhart-Hines *et al.* 2007). Thus, the use of CR or CR mimickers such as resveratrol or metformin can help to prevent the onset and the progression of these diseases.

CVD is one of the main problems in humans associated to ageing by accumulation of factors that impair cardiovascular function such as hypercholesterolaemia, high glucose or endothelial dysfunction. A 20% CR for 2–6 years in humans has been associated with lower levels of many of CVD markers and diabetes such as body weight, blood pressure, and blood cholesterol and glucose (Everitt & Le Couteur, 2007). But, as CR in humans is a complex and difficult therapy, exercise can be used as a mimetic. In fact, physical activity has shown positive effects by increasing lifespan without CVD (Franco *et al.* 2005) and lowering mortality risk in active subjects (Landi *et al.* 2004). Most of the mechanisms that maintain cardiac activity such as autophagy, proteasome-mediated turnover, apoptosis and control of quality of mitochondria

are affected during ageing and are improved by both CR and exercise (Quarles *et al.* 2015). The control of oxidative damage in plasma cholesterol, especially in LDL is another factor to be considered. We have recently demonstrated that increased physical activity is associated with lower oxidative damage in plasma of elderly people, probably through the increase in coenzyme Q in plasma lipoproteins, whereas sedentarism reduces coenzyme Q levels, which is associated with an increase in lipid peroxidation in plasma (Del Pozo-Cruz *et al.* 2014a,b). Similar results were obtained in mice forced to perform exercise (Rodríguez-Bies *et al.* 2015). These studies indicate that exercise and CR exerts a general effect and improves CVD not only by modulating heart and endothelial physiology but also reducing the oxidation of lipids in plasma and thus the production of the atherosclerotic plaque.

Concluding remarks

The broad effect of CR on healthspan and longevity occurs through multiple mechanisms that involve most of the metabolic pathways in tissues and organs (Fig. 4). The major effectors are sirtuin deacetylases, AMPK and PGC-1 α . CR improves aerobic metabolism by increasing efficient mitochondrial metabolism, lowering endogenous ROS production at the same time as it increases the amount and activity of endogenous antioxidant enzymes. These molecular and physiological effects have also been found with some nutraceuticals and compounds that act as CR mimetics such as resveratrol, rapamycin or metformin. CR also affects the lipid composition of membranes by lowering oxidative damage. Further, the study of the mechanisms involved in the prevention of chronic inflammation induced by CR, probably through similar mechanisms to those found in mitochondrial regulation, is increasing and offers new opportunities to understand how CR prevents endogenous damage in the organism.

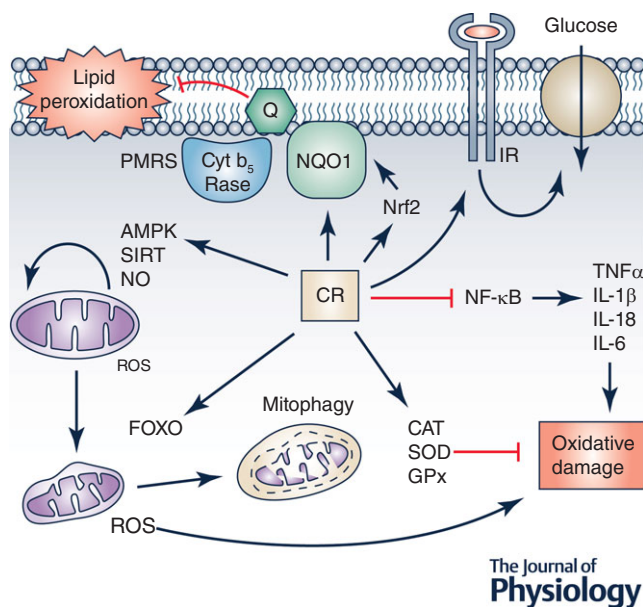


Figure 4. Resumé of CR effects on cells

CR induces many pathways that are involved in changes in mitochondrial physiology by activating AMPK- and SIRT-dependent pathways and inducing mitochondrial turnover by balancing mitochondrial biogenesis with induction of mitophagy of damaged mitochondria. This regulation reduces the production of ROS by damaged mitochondria, thus reducing oxidative damage. This damage is also reduced by the induction of cell antioxidant activities such as CAT, SOD and GPx and by preventing the activation of proinflammatory processes by blocking the NF κ B pathway. On the other hand, the role of plasma membrane redox system regulation in the CR effect on cells remains to be clarified but it seems that in aged cells and tissues the induction of this system by activation of Nrf2 not only prevents lipid peroxidation in membranes but can also regulate the response to external factors such as local factors or insulin. We cannot discard a putative role of this system in the regulation of the insulin response preventing insulin-resistance in aged cells.

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Additional information

Competing interests

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