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MINIREVIEW

Reactive oxygen species, ageing and the hormesis police

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Abstract

For more than 50 years, the free radical theory served as the paradigm guiding most investigations of ageing. However, recent studies in a variety of organisms have identified conceptual and practical limitations to this theory. Some of these limitations are related to the recent discovery that caloric restriction and other experimental manipulations promote longevity by inducing hormesis effects in association with increased reactive oxygen species (ROS). The beneficial role of ROS in lifespan extension is consistent with the essential role of these molecules in cell signalling. However, the identity of specific forms of ROS that promote longevity remains unclear. In this article, we argue that in several model systems, hydrogen peroxide plays a crucial role in the induction of hormesis.

Introduction

Hormesis is an adaptive response to a variety of oxidative and other stresses that renders cells/organisms resistant to higher (and normally harmful) doses of the same stressing agent (Martins *et al.*, 2011). The hormesis effects of reactive oxygen species (ROS) and their impact on ageing have emerged as one of the most important topics for understanding ageing as reflected in a number of recently published review articles (Hekimi *et al.*, 2011; Ristow & Schmeisser, 2011; Calabrese, 2012; Labunskyy & Gladyshev, 2012; Maryanovich & Gross, 2013). This review will address the question of the specific form of ROS that trigger hormesis. Recently, we established that hydrogen peroxide as a hormesis trigger and extend chronological lifespan (CLS) in budding yeast (Mesquita *et al.*, 2010). Nevertheless, more recent work on hormesis claims that superoxide anions are the triggers of hormesis effects. In this review, we decided to 'bring the case to the court' and to present the evidence supporting superoxide anions or hydrogen peroxide as triggers of hormetic effects. Arguments will be presented showing that hydrogen peroxide may be a general inducer of hormesis effects downstream of the superoxide anions through their conversion to hydrogen peroxide by superoxide dismutases. The evidence that hydrogen peroxide may induce quiescence, which is emerging as an important determinant of longevity, will be also discussed.

ROS as 'bad guys' – guilty as charged?

In the never-ending battle between processes that maintain cellular homoeostasis and factors that disrupt this equilibrium, ROS usually have been viewed as 'bad guys'. The deleterious effects of ROS are particularly relevant to the longstanding 'free radical theory of ageing', which posits that oxidative stress and oxidative damage to macromolecules are primary determinants of ageing. Despite the prominent position this theory has attained in more than 50 years of efforts to understand ageing, as has also been noted by others recently (Lapointe & Hekimi, 2010; Hekimi *et al.*, 2011), mounting evidence suggests that this theory is at best incomplete.

It has long been recognized that ROS cause complex and irreversible damage to cellular constituents that impair cellular homoeostasis (reviewed in de Grey, 2006). Oxidative damage is related to the high reactivity of molecular oxygen and its intermediates, which can lead

to oxidative modifications of proteins, lipids and DNA. One of the primary sources of ROS is mitochondria, and ROS damage to mtDNA has been implicated in a number of pathological conditions, including ageing. mtDNA damage can lead to aberrant mitochondrial function that can cause 'vicious cycles' of ROS production, which amplify damage. ROS-induced damage to mitochondria eventually became the basis for reformulation of this theory as the 'mitochondrial free radical theory'.

The results of numerous studies performed over a period of decades provide support for the free radical theory. For example, oxidatively damaged molecules accumulate in aged postmitotic cells in species as diverse as yeast and humans. These molecules include misfolded proteins, protein carbonyls and lipofuscin all of which slowly accumulate with age. Lipid peroxidation also increases with age and disrupts cellular functions occurring in biomembranes. A role for oxidative damage to DNA in ageing is consistent with accumulating evidence that genome instability and the rate at which this instability occurs increase with age. The free radical theory of ageing is also consistent with numerous studies in a variety of organisms showing that inhibition of antioxidant defences shortens lifespan. Additional support for the theory is provided by the observation that ROS inhibit telomerase and promote telomere erosion, a well-recognized component of ageing in eukaryotes (Passos *et al.*, 2007a). In the aggregate, the evidence that ROS and oxidative damage are important factors in ageing is compelling in many respects.

The case against the 'free radical theory of ageing'

In spite of the compelling evidence that ROS are guilty of disrupting cellular homoeostasis, in recent years, major cracks have appeared in the defence of the free radical theory. Evidence against the free radical theory has surfaced in studies of a variety of model organisms employed to investigate ageing. For example, we have reported the surprising observation that in the model organism *Saccharomyces cerevisiae* (budding yeast), hydrogen peroxide promotes chronological longevity, which is defined as the length of time that yeast cells survive in stationary phase cultures. The longevity effects of hydrogen peroxide occur in response to caloric restriction or inactivation of catalases and in cells ectopically exposed to low levels of hydrogen peroxide (Mesquita *et al.*, 2010). Our experiments provided additional evidence against the free radical theory as well – the longevity phenotype of catalase mutants was accompanied by elevated damage in the form of protein carbonyls and lipofuscin (Mesquita *et al.*, 2010). Similarly, elevated intracellular levels of ROS induced by 2-deoxy-D-glucose, which mimics caloric restriction by inhibiting glucose metabolism, were reported to extend the lifespan of worms (*Caenorhabditis elegans*; Schulz *et al.*, 2007; Hekimi *et al.*, 2011). Impaired insulin/IGF-1 signalling was also recently reported to induce a transient increase in ROS that extends worm lifespan (Zarse *et al.*, 2012). These reports are relevant to the well-established role that growth signalling by glucose and other factors plays in ageing in all organisms.

Additional evidence against the free radical theory was provided by the observation that exposure of long-lived *age-1* worms, but not wild-type worms, to high oxygen tension extends lifespan in parallel with decreased mitochondrial ROS and fewer protein carbonyls (Yanase *et al.*, 2002). These findings imply that the longevity effects of high oxygen in *age-1* worms are related to an initial increase in superoxide levels. Increased levels of superoxide anions have also been implicated in longevity effects in wild-type worms. For example, the lifespan of wild-type worms is extended by exposure to low concentrations of the superoxide-generating compound juglone (Cypser & Johnson, 2002). Elevated superoxide anion levels induced by mutations in the *nuo-6* and *isp-1* genes encoding subunits of complex I and III of the respiratory chain or by exposing worms to the superoxide-generating compound paraquat also extend the lifespan of wild-type worms (Yang & Hekimi, 2010). The absence of an effect in wild-type worms exposed to hyperoxic conditions may reflect an upper limit of superoxide anions that promote longevity, which may have been exceeded in these conditions. These and other findings recently reported in studies of more complex organisms (Perez *et al.*, 2009) argue for acquittal of the charges against ROS brought by the free radical theory as 'bud guys' that promote ageing.

The verdict: guilty of some, but not all charges

Although hydrogen peroxide enhances chronological lifespan (CLS) in calorie-restricted budding yeast cells, this occurs in parallel with a decrease in superoxide anions. This decrease is caused by induction by hydrogen peroxide of the activity of the cytosolic Cu/Zn-dependent and the mitochondrial Mn-dependent superoxide dismutases, Sod1p and Sod2p, respectively (Mesquita *et al.*, 2010). The induction of SOD activity by hydrogen peroxide is consistent with the earlier demonstration that budding yeast CLS is extended by SOD overexpression (Fabrizio *et al.*, 2004; Harris *et al.*, 2005). These findings are also consistent with earlier reports that sublethal concentrations of hydrogen peroxide induce transcription of both the *SOD1* and *SOD2* genes (Godon *et al.*, 1998; Gasch *et al.*, 2000) as well as an increase in levels of the corresponding proteins (Godon *et al.*, 1998). Transactivation of

SOD1 and *SOD2* is mediated in part by the transcriptional regulator Yap1p (Yahara, 1996). It is well documented that hydrogen peroxide modifies Yap1p as part of a regulatory mechanism associated with Yap1p induction of a large number of genes (Delaunay *et al.*, 2000, 2002).

The induction of superoxide dismutase transcription and/or activity by hydrogen peroxide is a highly conserved regulatory mechanism. For example, hydrogen peroxide induces sodA transcription in *Escherichia coli* (Semchyshyn, 2009), as well as the transcription and activity of MnSOD in rat cells (Yoshioka *et al.*, 1994). Recent data, obtained in *S. cerevisiae*, also suggest that not only the interaction between hydrogen peroxide and cellular proteins plays a role in signalling transduction but also thiol peroxidases can sense and transfer oxidative signals to signalling proteins regulating transcription (Fomenko *et al.*, 2011). Thus, not only the regulatory mechanism of hydrogen peroxide seems to be conserved, but also its role in promoting longevity may be conserved as well. Consistent with this possibility, the replicative lifespan of human skin keratinocytes is extended by ectopic exposure to low concentrations of hydrogen peroxide (Yokoo *et al.*, 2004). This effect is accompanied by an increase in telomere length (Yokoo *et al.*, 2004). In mammalian cells, telomere elongation is inhibited by superoxide anions (Passos *et al.*, 2007b), and telomere maintenance is enhanced by SOD activity (Serra *et al.*, 2003). It is possible, therefore, that the replicative lifespan-extending effect of hydrogen peroxide in human keratinocytes is partially related to hydrogen peroxide-induced SOD activity that reduces superoxide anions.

A transient increase in intracellular hydrogen peroxide has also been implicated in lifespan extension associated with impaired insulin/IGF-1 signalling in *C. elegans*, which disrupts glucose uptake and up regulates superoxide dismutases and other oxidative stress defences, leading to a subsequent decline in overall levels of ROS (Zarse *et al.*, 2012). Increased hydrogen peroxide also underlies the pro-survival effects of inactivating catalases or ectopic exposure to hydrogen peroxide in mutant *C. elegans* dauer larvae (Xie & Roy, 2012). The beneficial effects of hydrogen peroxide reported in this latter study were attributed to the induction of HIF-1-dependent alterations in lipid metabolism. Elevated intracellular levels of hydrogen peroxide may also underlie the switch from short-lived 'worker' ants to longer-lived reproductive gamergates in the eusocial insect *Harpegnathos saltator*. This switch is accompanied by a substantial decrease in catalase activity, which is expected to elevate intracellular levels of hydrogen peroxide (Schneider *et al.*, 2011).

The longevity-promoting effects of caloric restriction induced by hydrogen peroxide in stationary phase budding yeast cells are accompanied by an enhanced quiescence state indicated by the reduced frequency of budded cells (Weinberger *et al.*, 2007, 2010). This reflects the less frequent entry of calorie-restricted stationary phase cells into S phase (Weinberger *et al.*, 2013), where they suffer replication stress (Murakami *et al.*, 2012; Weinberger *et al.*, 2013). Even in the absence of caloric restriction, hydrogen peroxide levels increase in parallel with the reduction in superoxide levels as budding yeast cells approach stationary phase (Mesquita *et al.*, 2010; Pan *et al.*, 2011). This suggests that the regulation of SODs by hydrogen peroxide is a general feature of quiescence in budding yeast that is enhanced by caloric restriction. A similar reciprocal relationship between increased levels of hydrogen peroxide and decreased levels of superoxide anions has been reported in cultured mouse cells as they approach quiescence due to contact inhibition (Sarsour *et al.*, 2008). Furthermore, disrupting this relationship by inactivating MnSOD, which elevates levels of superoxide and decreases levels of hydrogen peroxide, inhibits quiescence as indicated by a higher fraction of cells that remained in S phase followed by cell death (Sarsour *et al.*, 2008). Quiescence is also emerging as an important component of longevity in mammalian cells (Chakkalakal *et al.*, 2012). Although a causal role for hydrogen peroxide in maintaining quiescence in mammalian cells has not been addressed, the studies described above clearly suggest this possibility.

Hydrogen peroxide and/or superoxide anions – the hormesis police?

All of these findings point to superoxide anions as a guilty party in the loss of cellular homoeostasis that underlies ageing and to low levels of hydrogen peroxide as the 'sheriff' that keeps superoxide anions in check. The critical role of hydrogen peroxide in policing levels of superoxide anions conforms to current concepts of a phenomenon called hormesis (Calabrese, 2012). However, the results of some studies suggest that not all superoxide anions are bad guys that need to be restrained by oxidative stress defences – similar to hydrogen peroxide, superoxide anions are also capable of enhancing lifespan (Fig. 1). This includes, for example, the longevity-promoting effect of hyperoxia in *age-1* worms, as well as the lifespan-extending effects in wild-type worms of the compounds juglone and paraquat and of mutations in the *nuo-6* and *isp-1* genes described above (Yang & Hekimi, 2010). In budding yeast, inactivation of TOR signalling by deletion of the *TOR1* gene extends CLS by increasing superoxide anions early in CLS experiments (Pan *et al.*, 2011). Consistent with a role for superoxide anions in lifespan extension of *tor1Δ* cells, the superoxide-generating compound menadione mimics the longevity effects of

TOR1 inactivation. Also consistent with this model, uncoupling of mitochondrial respiration from oxidative phosphorylation with dinitrophenol, which inhibits the production of superoxide anions, also attenuates the lifespan-extending effect of *TOR1* deletion (Pan *et al.*, 2011).

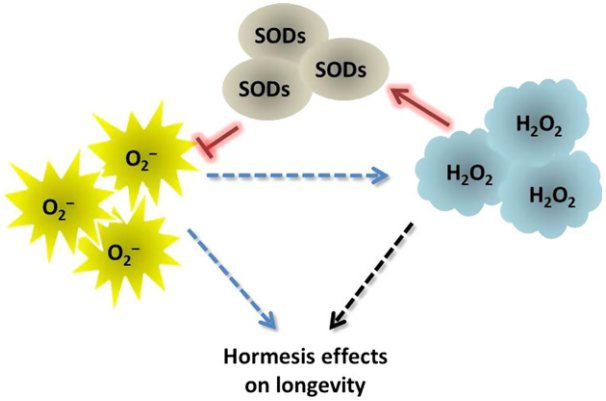

Fig. 1. Both hydrogen peroxide and superoxide anions have been implicated in hormesis effects that promote longevity. Whether superoxide anions can induce hormesis effects directly or indirectly (blue dashed arrows) via their conversion into hydrogen peroxide by SODs remains unclear. Importantly, the levels of superoxide anions and hydrogen peroxide are regulated by SOD activity. Hydrogen peroxide increases and promotes SOD activity that decreases superoxide levels (red arrows), thus creating a feedback regulatory mechanism that promotes hormesis effects by maintaining hydrogen peroxide at homoeostatic levels.

The lifespan-extending effects of ectopic exposure to hydrogen peroxide detected in budding yeast (Mesquita *et al.*, 2010) and worms (Xie & Roy, 2012) definitively establish this form of ROS as a *bona fide* inducer of hormesis. Although it has been suggested that the effects of ROS on lifespan are conceptually quite distinct from hormesis effects (Hekimi *et al.*, 2011), the effects of hydrogen peroxide on lifespan were detected in parallel with adaptive responses that elevated SOD activity (Mesquita *et al.*, 2010) or altered lipid metabolism (Xie & Roy, 2012), which clearly meets the definition of hormesis. It is important to keep in mind that one source of intracellular hydrogen peroxide is the dismutation of superoxide anions by SODs. Therefore, the hormesis effects of superoxide anions reported in some of the studies cited above may in fact be triggered by hydrogen peroxide produced from superoxide anions by SOD activity (Fig. 1). Consistent with this possibility, caloric restriction induced by switching exponentially proliferating cultures to a lower concentration of glucose at the beginning of yeast chronological ageing experiments induces an initial increase in superoxide anions (Weinberger *et al.*, 2010), which is followed by the sustained increase in hydrogen peroxide that triggers SOD activity and reduced levels of superoxide

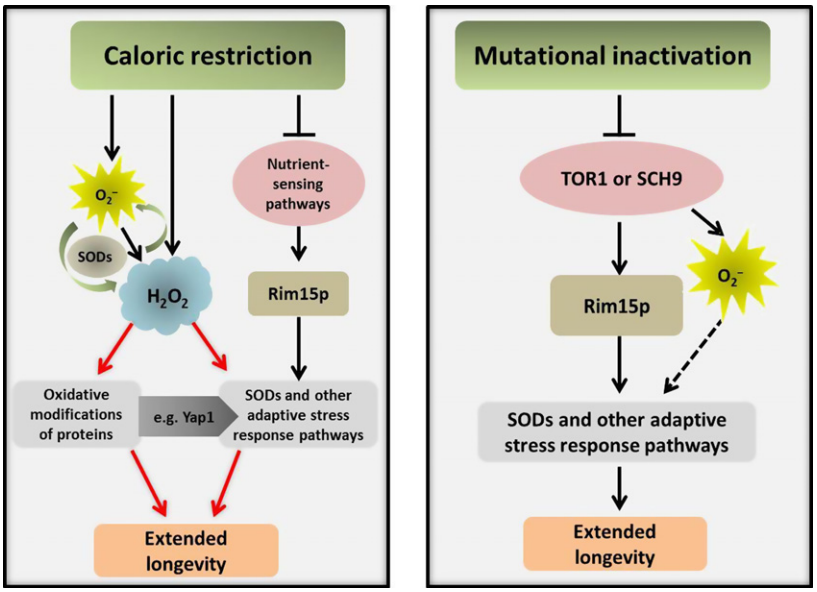

Fig. 2. Pathways mediating hormesis extension of CLS in budding yeast triggered by caloric restriction or mutational inactivation of growth signalling pathways. Dotted lines represent hypothetical pathways. Although O_2^- is dismutated to H_2O_2, whether this is the source of H_2O_2 in calorie-restricted cells has not yet been tested and other potential sources exist, such as peroxisomes. Hydrogen peroxide modification of the transcriptional activator Yap1p (Georgiou, 2002) likely contributes to the induction of SODs (Mesquita *et al.*, 2010) and other adaptive stress response pathways. It is likely that H_2O_2 also induces oxidative stress responses by modifying other proteins, including those regulating epigenetic effects that also contribute to longevity, as described in mammalian cells (reviewed in Maryanovich & Gross, 2013). Although indirect evidence suggests that O_2^- triggers oxidative stress defences when Tor1p-Sch9p signalling is mutational inactivated, this possibility also has not been tested directly.

anions (Mesquita *et al.*, 2010). The early burst of superoxide anions induced by caloric restriction in these experiments likely reflects an increase in respiration rate first reported in calorie-restricted cells several years ago (Lin *et al.*, 2002).

However, the increased CLS triggered by inactivating *TOR1* is accompanied by a decrease, rather than increase, in hydrogen peroxide levels (Pan *et al.*, 2011). A decrease in intracellular hydrogen peroxide also occurs in concert with chronological lifespan extension in stationary phase cells when *SCH9*, a downstream effector of *TOR1* signalling, is inactivated (Weinberger *et al.*, 2010). Similarly, the increase in superoxide anions in long-lived *nuo-6* and *isp-1* mutant worms described above was detected in the absence of an increase in hydrogen peroxide or other forms of ROS (Yang & Hekimi, 2010). These results point to superoxide anions as a different branch of the hormesis police that extends lifespan in the absence of hydrogen peroxide effects.

The specific forms of ROS that trigger hormesis effects in laboratory experiments may be related to how specific experimental manipulations impact a myriad of signalling pathways that interact with one another in complex ways. In fact, although a number of antioxidant and repair processes have been characterized, the mechanisms whereby cells are damaged by particular oxidants or protect themselves from damage remain to be identified (Thorpe *et al.*, 2004).

The parallels between hormetic effects of caloric restriction or mutational inactivation of growth signalling pathways in budding yeast chronological ageing experiments may be informative in this regard (Fig. 2). Caloric restriction extends CLS in part by attenuating Tor1p-Sch9p-dependent glucose signalling, which leads to the activation of Rim15p and oxidative stress defences regulated by this transcriptional activator (Wei *et al.*, 2008). As noted above, caloric restriction also increases intracellular hydrogen peroxide, which also induces oxidative stress defences (Mesquita *et al.*, 2010), but independently of Rim15p (Weinberger *et al.*, 2010). The increase in hydrogen peroxide in calorie-restricted cells may be triggered by the increase in superoxide anions that occurs before cells transition into stationary phase (Weinberger *et al.*, 2010), although other possible sources of hydrogen peroxide exist. Mutational inactivation of Tor1p-Sch9p (Fig. 2) also induces Rim15p-dependent oxidative stress defences, as well as an increase in intracellular superoxide anions, which may occur independently of Rim15p. This increase may induce SODs and other oxidative stress defences and/or altered mitochondrial function (Pan *et al.*, 2011). Ultimately, the effects of both types of experimental manipulations converge on a reduction in superoxide anions in cells in stationary phase that promotes longevity (Fig. 2).

In the context of these parallels and the fact that both types of experimental manipulations result in reduced Tor1p-Sch9p signalling, it is puzzling that mutational activation of Sch9p or Tor1p does not lead to an increase in hydrogen peroxide, similar to caloric restriction. Apparently, in addition to down regulating Tor1p and Sch9p signalling, caloric restriction triggers additional events that increase intracellular levels of hydrogen peroxide. We propose that this occurs as part of a mechanism that amplifies the hormetic effects of mutationally inactivating a subset of signalling pathways in laboratory experiments. Outside the laboratory, the depletion of nutrients likely triggers in yeast and other organisms a deeper and more sustained response that requires hydrogen peroxide to efficiently drive cells into and maintain a quiescent state, where cells are protected from deleterious effects of superoxide anions and a paucity of nutrients on DNA replication (Weinberger *et al.*, 2010). According to this model, superoxide anions have been justly convicted as the bad guys, although the initial increase in their numbers triggered by caloric restriction may alert cells to the need to round them up. This becomes the job of hydrogen peroxide, which may serve as the true hormesis police that promotes longevity in organisms faced with stresses that more closely resemble those they encounter in their natural environment. Future studies will determine whether this verdict is corrected.

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