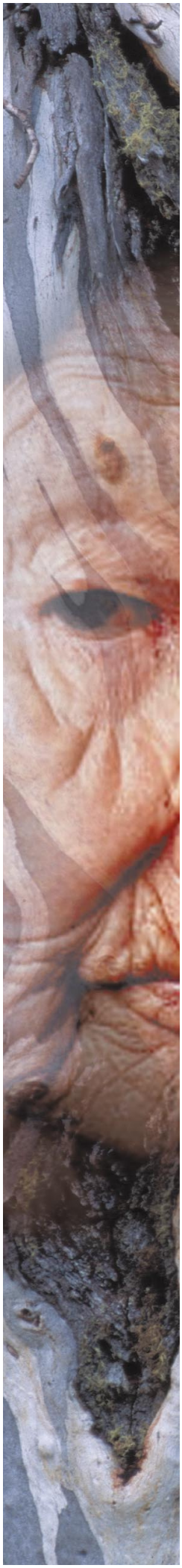

Oxidants, oxidative stress and the biology of ageing

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Living in an oxygenated environment has required the evolution of effective cellular strategies to detect and detoxify metabolites of molecular oxygen known as reactive oxygen species. Here we review evidence that the appropriate and inappropriate production of oxidants, together with the ability of organisms to respond to oxidative stress, is intricately connected to ageing and life span.

Nearly a century ago it was noted that animals with higher metabolic rates often have shorter life spans. These observations led to the formulation of 'the rate-of-living hypothesis', which states that the metabolic rate of a species ultimately determines its life expectancy. Initially, the mechanistic link between metabolism and ageing was unknown. In the mid-1950s, Denham Harman articulated a 'free-radical theory' of ageing, speculating that endogenous oxygen radicals were generated in cells and resulted in a pattern of cumulative damage[1]. Although the concept of endogenous oxidants was at first controversial, the identification a decade later of superoxide dismutase (SOD)[2], an enzyme whose sole function seemed to be the removal of superoxide anions, provided mechanistic support for Harman's hypothesis. Given that the mitochondria produce most of the energy in the cell, and correspondingly consume the bulk of intracellular oxygen, the free-radical theory of ageing is now often thought of synonymously with the rate-of-living hypothesis; the higher the metabolic rate of an organism, the greater the production of reactive oxygen species (ROS) and hence the shorter the life span. However, in some species the strict correlation between metabolic rate and life span is not maintained. This is particularly true for birds and primates, which tend to live longer than would be predicted by their metabolic rates. Careful analysis of oxidant production demonstrated that at a given metabolic rate, mitochondria from these species tend to produce fewer ROS[3]. This indicates that ROS production rather then metabolic rate provides the strongest correlation with overall longevity.

The free-radical theory of ageing originally implied that the targets of ROS were random, indiscriminate and cumulative. However, although oxidants may certainly function stochastically, accumulating evidence implicates ROS as specific signalling molecules under both physiological and pathophysiological conditions. This more complex view of the importance of oxidants in biological processes is depicted in Fig. 1. As indicated, the generation of ROS, within certain boundaries, is essential to maintain homeostasis. For example, ROS generation by phagocytic cells constitutes an essential host defence mechanism necessary to combat infection. Likewise, cytosolic ROS produced in response to stimulation by growth factors are involved in regulating the proliferative response[4]. Under certain situations of metabolic stress, even mitochondrial-derived oxidants seem to function as signalling molecules[5,6]. Regardless of how or where they are generated, a rise in intracellular oxidant levels has two potentially important effects: damage to various cell components and triggering of the activation of specific signalling pathways. Both of these effects can influence numerous cellular processes linked to ageing and the development of age-related diseases. In this review, we address how ROS are generated, how the cell responds to oxidative stress and how these responses change with age. In addition, we describe the mounting genetic evidence that links oxidants and oxidative stress responsiveness to ageing and discuss the challenges associated with the potential development of anti-ageing therapies.

Oxidant production and antioxidant defences

For the purpose of this discussion, ROS encompass a variety of diverse chemical species including superoxide anions, hydroxyl radicals and hydrogen peroxide. Some of these species, such as superoxide or hydroxyl radicals, are extremely unstable, whereas others, like hydrogen peroxide, are freely diffusible and relatively long-lived. These various radical species can either be generated exogenously or produced intracellularly from several different sources. Cytosolic enzyme systems contributing to oxidative stress include, among others, the expanding family of NADPH oxidases, a superoxide-generating system that was first described in the neutrophil. The newly described non-phagocytic NADPH oxidases also generate superoxide anions, but depending on the specific NADPH oxidase expressed, can either trigger cellular transformation or replicative senescence[7,8]. The observation that different members of the NADPH oxidase family can have such widely differing biological outcomes reinforces the complexity in determining the cellular response to oxidants. Contributing factors may include the cell type, the absolute level and duration of oxidant production, the species of ROS generated, and the specific intracellular site of ROS production. Nonetheless, the NADPH oxidase family of enzymes, like the widely characterized nitric oxide synthase (NOS) family, illustrates the apparent purposeful and deliberate use of oxidant generation in normal cellular signalling and homeostasis.

Most estimates suggest that the majority of intracellular ROS production is derived from the mitochondria. The production of mitochondrial superoxide radicals occurs primarily at two discrete points in the electron transport chain, namely at complex I (NADH dehydrogenase) and at

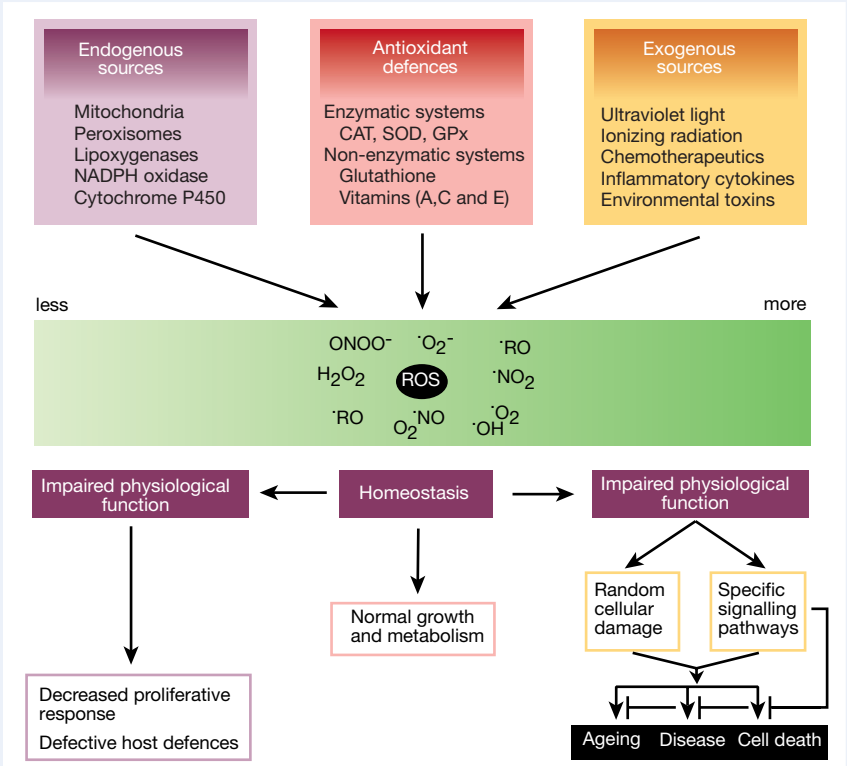

Figure 1 The sources and cellular responses to reactive oxygen species (ROS). Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, a number of external agents can trigger ROS production. A sophisticated enzymatic and non-enzymatic antioxidant defence system including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defence. Similarly, increased ROS may also be detrimental and lead to cell death or to an acceleration in ageing and age-related diseases. Traditionally, the impairment caused by increased ROS is thought to result from random damage to proteins, lipids and DNA. In addition to these effects, a rise in ROS levels may also constitute a stress signal that activates specific redox-sensitive signalling pathways. Once activated, these diverse signalling pathways may have either damaging or potentially protective functions.

complex III (ubiquinone–cytochrome *c* reductase). Under normal metabolic conditions, complex III is the main site of ROS production[9]. With respect to human ageing, the Achilles' heel of this elegant system lies in the formation of the free radical semiquinone anion species (·Q−) that occurs as an intermediate in the regeneration of coenzyme Q (Fig. 2). Once formed, ·Q− can readily and non-enzymatically transfer electrons to molecular oxygen with the subsequent generation of a superoxide radical. The generation of ROS therefore becomes predominantly a function of metabolic rate and, as such, the rate of living can be indirectly translated to a corresponding rate of oxidative stress. In addition to generating oxidants, metabolism can produce a host of other by-products including glyoxal and methylglyoxal, both of which can contribute to advanced glycation end-product (AGE) formation that, in turn, seems to contribute to the ageing phenotype[10].

Evidence indicates that, *in vitro*, mitochondria convert 1–2% of the oxygen molecules consumed into superoxide anions[11]. Given that these initial estimates were made on isolated mitochondria in the presence of high, non-physiological concentrations of oxygen, the *in vivo* rate of mitochondrial superoxide production is undoubtedly considerably less. Whatever the absolute amount of mitochondrial ROS, given their potentially harmful effects, it is likely that numerous protective mechanisms have evolved to limit oxidant production and release. A further understanding of such regulatory mechanisms may reveal potential targets for therapeutic intervention. One postulated mechanism to reduce mitochondrial oxidant production is to increase the rate of metabolic uncoupling[12]. When oxygen consumption is uncoupled from ATP generation, heat is produced. This thermogenesis is mediated by an expanding family of uncoupling proteins (UCP-1, -2 and -3). However, the consumption of oxygen without ATP production would also reduce the level of free molecular oxygen potentially available for superoxide anion formation (Fig. 2). Consistent with the hypothesis that metabolic uncoupling might regulate ROS release is the recent evidence indicating that an increase in uncoupling reduces mitochondrial ROS release[5], whereas levels of mitochondrial oxidants rise in mice with a targeted deletion of UCP-3 (ref. 13).

The burden of ROS production is largely counteracted by an intricate antioxidant defence system that includes the enzymatic scavengers SOD, catalase and glutathione peroxidase. SOD speeds the conversion of superoxide to hydrogen peroxide, whereas catalase and glutathione peroxidase convert hydrogen peroxide to water. In addition to these well characterized antioxidant enzymes, at least five members of a new family of peroxide scavengers termed peroxiredoxins have recently been isolated[14]. A variety of other non-enzymatic, low molecular mass molecules are important in scavenging ROS. These include ascorbate, pyruvate, flavonoids, carotenoids and perhaps most importantly, glutathione, which is present in millimolar concentrations within cells.

The balance between ROS production and antioxidant defences determines the degree of oxidative stress. Consequences of this stress include modification to cellular proteins, lipids and DNA. The most widely studied oxidative stress-induced modification to proteins is the formation of carbonyl derivatives[15]. Carbonyl formation can occur through a variety of mechanisms including direct oxidation of certain amino-acid side chains and oxidation-induced peptide cleavage. Although all organs and all proteins can potentially be modified by oxidative stress, certain tissues and specific protein targets may be especially sensitive[16,17]. A recent report indicated that protein misfolding, independent of the cellular redox state, increases protein carbonylation[18]. As such, the notion that the rate of carbonyl formation is always directly proportional to the degree of oxidative stress may need to be re-examined.

Several studies have shown that ageing cells and organisms accumulate increased levels of oxidant-damaged nuclear DNA[19]. Perhaps because of its proximity to the main source of oxidant generation, or because of a limited DNA repair system, mitochondrial DNA is generally considered to be even more sensitive then nuclear DNA to oxidative damage. Two recent studies provided direct proof that oxidative stress can induce mitochondrial DNA damage. In these studies, oxidative stress was genetically engineered by targeted deletions in either Mn-SOD or the adenine nuclear transporter. These knockout mice had a respective defect in mitochondrial

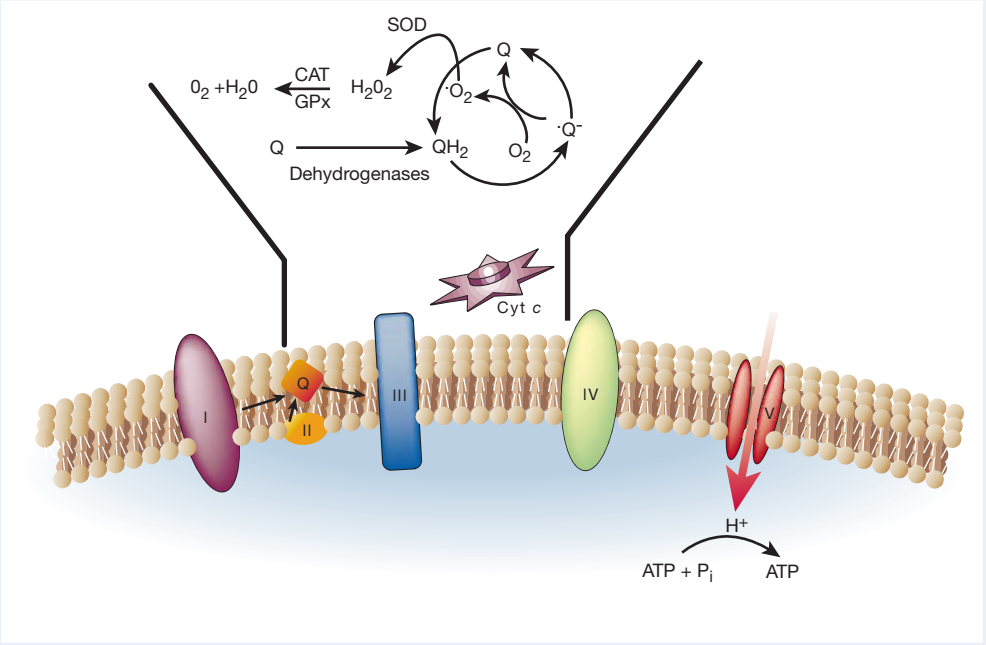

Figure 2 Complex III is the major source of mitochondrial ROS production. Electrons from complex I or II dehydrogenases are transfered to coenzyme Q (Q), also called ubiquinone. The resulting reduced form (QH_2) of coenzyme Q subsequently undergoes two sequential one-electron reductions (the Q cycle) using oxidized and reduced forms of cytochrome *b* and cytochrome *c* (Cyt *c*). The unstable intermediate in the Q cycle ($\cdot Q-$) can lead to superoxide formation by transferring electrons directly to molecular oxygen. Once generated, the superoxide can be enzymatically dismutated by SOD to form hydrogen peroxide that in turn is metabolized by enzymes such as catalase (CAT) and glutathione reductase (GPx) regenerating water and molecular oxygen. The generation of superoxide is non-enzymatic and hence the higher the rate of metabolism, the greater the production of ROS.

superoxide scavenging capacity or overall aerobic metabolism. Subsequent analysis of these animals showed significant increase in the level of mitochondrial DNA rearrangements[20,21]. Increasing damage to mitochondrial DNA inevitably leads to compromised mitochondrial function and integrity. Damaged mitochondria are thought to release more ROS and set in motion a vicious cycle of increasing DNA damage leading to increased ROS production that in turn leads to more DNA damage.

Cellular senescence and oxidative stress

After a finite number of divisions, primary cell cultures enter a state of replicative senescence in which they are growth-arrested and refractory to further mitogenic stimulation. Although the relevance of *in vitro* senescence to organismal ageing remains controversial, several studies indicate that oxidants are important in the development of the senescent phenotype. Early studies with human diploid fibroblasts revealed that cells grown in low oxygen tension exhibit a prolonged life span[22]. In contrast, cells grown in the presence of high oxygen concentrations have a reduced life span and show an accelerated rate of telomere shortening per population doubling[23]. Similarly, treatment of cultures of primary fibroblasts with moderate, non-lethal doses of exogenous hydrogen peroxide activates a rapid, senescence-like growth arrest[24].

The role of oxidants in cellular senescence was further underscored by recent observations that overexpression of an activated Ras gene can also induce a senescence-like state in human diploid fibroblasts[25]. Subsequent analysis demonstrated that expression of activated Ras in diploid fibroblasts resulted in an increase in oxidant levels[26]. In addition, although expression of activated Ras in human diploid fibroblasts produced growth arrest, this arrest could be reversed either by reducing ambient oxygen or by treatment with a cell-permeable antioxidant. As such, these results raise the possibility that a moderate, sustained rise in oxidants may function as a common trigger for activation of the senescence programme.

Oxidants in cellular signalling

Although the preceding discussions have focused mainly on the endogenous generation of ROS as a consequence of metabolic activities, many environmental stimuli including cytokines, ultraviolet (UV) radiation, chemotherapeutic agents, hyperthermia and even growth factors generate high levels of ROS that can perturb the normal redox balance and shift cells into a state of oxidative stress. When the stress is severe, survival is dependent on the ability of the cell to adapt to or resist the stress, and to repair or replace the damaged molecules. Alternatively, cells may respond to the insult by undergoing apoptosis, a process whereby severely damaged cells are removed from the multicellular host, and which, within limits, preserves the organism. A number of stress response mechanisms have evolved to help the cell and organism adapt to acute stress, and acting in either a cooperative or antagonistic fashion they serve to coordinate the acute cellular stress response and ultimately determine the outcome. Many of these pathways have been faithfully preserved throughout evolution. Thus, both the stresses and the response mechanisms represent players in an ancient battle. As will be discussed later, many mutations that prolong life seem to provide a global increase in stress resistance. Therefore, a more complete understanding of the cellular response to stress should provide significant insight into ageing.

Among the main stress signalling pathways and/or central mediators activated in response to oxidant injury are the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signalling cascades, the phosphoinositide 3-kinase (PI(3)K)/Akt pathway, the nuclear factor (NF)-κB signalling system, p53 activation, and the heat shock response (Fig. 3). Activation of these pathways is not unique to oxidative stress, as they are known to have central roles in regulating cellular responses to other stresses as well as regulating normal growth and metabolism. Indeed, in some situations the response to oxidants may involve overstimulation of normal ROS-regulated signalling pathways. In general, the heat shock response, ERK, PI(3)K/Akt and NF-κB signalling pathways exert a pro-survival influence during oxidant injury, whereas activation of p53, JNK and p38 are more commonly linked to apoptosis. However, numerous exceptions to these generalities can be found.

The initiating events leading to activation of pathways in response to oxidants are incompletely understood. In the case of p53 activation, oxidative stress may be sensed as a consequence of the DNA damage it causes. However, in some cells, elevated p53 expression results in increased oxidative stress[27,28], suggesting that an important consequence of oxidant-induced p53 activation is a further increase in the level of oxidative stress. This positive feedback loop may be important in committing to an apoptotic response.

Oxidants seem to activate the ERK and the PI(3)K/Akt pathways largely through stimulation of growth-factor receptors, mimicking

Table 1 **Mutations revealing links between longevity and stress resistance in multicellular organisms**

Species/mutations	Gene description	Phenotypic influence* Life span†	Stress resistance
C. elegans			
age-1	Human PI(3)K homologue	65% increase	Enhanced (UV, paraquat, heat)
daf-2	Human insulin-receptor homologue	100% increase	Enhanced (UV, paraquat, heat)
daf-16	Forkhead transcription factor	Suppresses longevity conferred by *age-1* and *daf-2* mutations	Suppresses stress resistance of *age-1* and *daf-2* mutants
clk-1	Homologue of yeast gene associated with coenzyme Q biosynthesis	40% increase	Enhanced increased (UVC)
spe-10	Unknown (sperm defective)	40% increase	Enhanced (UV, paraquat, but not heat)
spe-26	Unknown (sperm defective)	65% increase	Enhanced (UV, paraquat, heat)
old-1	Putative receptor tyrosine kinase	65% increase	Enhanced (UV, heat)
ctl-1	Cytosolic catalase	25% decrease; suppresses longevity conferred by *daf-2*, *age-1* and *clk-1*	Not determined
mev-1	Cytochrome *b* subunit of succinate dehydrogenase	37% decrease	Hypersensitive to oxygen
Drosophila			
mth	Putative G-protein-coupled receptor	35% increase	Enhanced (UV, paraquat, heat)
Mouse			
shc^{66}	Cytoplasmic signal-transduction adaptor protein	30% increase	Enhanced (UV, H_2O_2)

*See ref. 83 for original references describing the phenotypes of the *daf-2*, *daf-16*, *clk-1* and *spe-26* mutants. References for other mutants are as follows: *age-1* (refs 49, 50, 83); *spe-10* (ref. 84); *old-1* (ref. 85); *ctl-1* (ref. 56); *mev-1* (ref. 55); *mth* (ref. 60); and shc^{66} (ref. 68).
†Numbers reflect changes in mean life span of the mutants relative to wild-type animals.

the actions of natural ligands. Many growth-factor receptors have been shown to undergo enhanced phosphorylation in response to direct treatment with oxidants, and agents or conditions that prevent receptor phosphorylation likewise inhibit the activation of ERK and Akt by oxidants[29–31]. One mechanism proposed to explain this effect is oxidant-mediated inactivation of critical phosphatases necessary for dephosphorylation (turning off) of the growth-factor receptors[31]. Support for such a mechanism has come from the finding that hydrogen peroxide, either derived exogenously or produced endogenously after growth-factor stimulation, can reversibly inactivate protein-tyrosine phosphatase 1B in cells[32]. The activation of growth-factor-receptor signalling pathways by oxidants is consistent with the demonstration that low concentrations of exogenous hydrogen peroxide are mitogenic[33].

Oxidative stress might induce activation of the JNK and p38 kinase pathways by an additional mechanism. The redox regulatory protein thioredoxin (Trx) has been shown to bind to apoptosis signal-regulating kinase (ASK1), an upstream activator of both JNK and p38, and under normal conditions inhibit its activity[34]. However, oxidative stress causes dissociation of the Trx–ASK1 complex and subsequent activation of the downstream JNK and p38 kinase[34]. Similarly, biochemical evidence indicates that under non-stressed conditions glutathione *S*-transferase binds to JNK to inhibit its activation, but that this interaction is also disrupted by oxidative stress[35]. These results show an intimate coupling between alterations in the intracellular redox state and the activity of downstream stress-activated pathways. The observation that multiple pathways are sensitive to a rise in ROS levels indicates that these pathways may have evolved, in part, to allow organisms to survive within an aerobic environment. In addition, it suggests that a rise in ROS might represent a common, if not universal, signal of cellular stress.

A common effect of the activation of these pathways is a change in pattern of gene expression mediated largely through modulation of the activities of transcription factors. Accordingly, a large number of oxidative stress-responsive transcription factors and genes have been identified[36] and some of these have been implicated in influencing ageing processes. The effect of ROS on expression and activity of transcription factors is complex and occurs at multiple levels, often in a seemingly antagonistic or paradoxical fashion. For example, although ROS generally cause an increase in AP-1 levels and increased nuclear translocation of NF-κB, oxidant stress can at the same time reduce the transcriptional activity of these molecules through the direct oxidation of critical cysteine residues contained within the DNA-binding domain[36].

Analysis of the integrated cellular response to oxidative stress is an area that is particularly suited for analysis using a genomic or proteomic approach. Initial characterization using the latter technique in peroxide-challenged yeast revealed over a 100 proteins whose levels changed after oxidative stress[37]. As might be expected, included among these were proteins involved in scavenging ROS, as well as heat shock proteins and chaperones. Nearly a quarter of the proteins identified were involved in carbohydrate metabolism. In general, oxidative stress repressed a number of proteins involved in glycolysis and the tricarboxylic acid cycle. This was interpreted as an attempt to restore levels of reducing equivalents such as NADPH at the expense of ATP generation. If these results are confirmed in higher eukaryotes it would imply that ROS may not only be by-products of metabolism but also be regulators of metabolic rates. Such a concept further blurs the distinction between the rate-of-living hypothesis and the free-radical theory of ageing and raises the possibility that the rate of living may determine the level of oxidant generation and that oxidant generation may in turn modulate the rate of living.

Several of the pathways activated by acute oxidative stress show diminished activity as a function of ageing. In a variety of model systems and stress paradigms including oxidative stress, the magnitude of induction of heat-shock proteins, and Hsp70 in particular, is attenuated with age[38]. The heat-shock protein family encompasses many chaperones involved in regulating the folding, transport and degradation of other cellular proteins. The relationship between ageing and a decline in the robustness of this stress response is unclear, but evidence indicates that elevating levels of Hsp70 enhances the survival of stressed cells and/or animals, whereas inhibiting this stress response reduces survival[39].

In contrast to the age-related attenuation in heat-shock protein induction in response to acute stress, two recent studies using complementary DNA microarray analysis to examine global age-associated changes in gene expression in mouse tissues provided evidence that basal expression (that seen in the absence of overt stress) of certain heat-shock proteins actually increases with ageing[40,41]. This elevated expression (which involves members other than Hsp70) was interpreted to occur as a response to the age-associated accumulation of oxidatively damaged proteins. Age-associated elevations in expression of heat-shock proteins have also been observed in *Drosophila*[42] and *Caenorhabditis elegans* (T. Johnson, personal communication). In some systems, stress-induced activation of the

ERK signalling pathway is also attenuated with ageing[43]. Like the heat-shock response, ERK activation exerts a pro-survival signal during oxidative stress[44]; thus, reduced ERK activity in aged cells may have a negative impact on survival. It is also worth noting that activation of ERK in response to mitogenic stimulation is also reduced as a function of ageing[45], again emphasizing the intimate link between proliferative and oxidative stress-response pathways. Basal DNA-binding activity of NF-κB has been shown to increase with age, which again has been suggested to reflect increased oxidative stress in aged cells and tissues[46,47]. However, as with heat-shock protein expression, evidence indicates that acute activation of this transcription factor by extracellular signals in T cells is diminished with ageing[48].

Genetics, oxidative phenomena and longevity

If oxidative stress and the ability to respond appropriately to it is important in ageing, then it follows that factors that increase resistance to stress should have anti-ageing benefits and lead to enhanced life span. In support of this claim, genetic links between stress responsiveness and longevity have been established in *C. elegans*, *Drosophila* and mice (Table 1). In *C. elegans*, such a relationship between life span and stress resistance was first demonstrated for *age-1* mutants which also displayed age-dependent elevations in CuZn-SOD and catalase activities[49,50]. A variety of other life-extending mutations that are likewise correlated with enhanced stress tolerance have since been described (see Table 1 for references).

To the extent that their functions are known, many of the mutated genes encode proteins involved in regulating energy use, and therefore potentially the level of ROS. For example, the *age-1*, *daf-2* and *daf-16* genes in *C. elegans* are associated with an insulin-like signalling pathway that regulates dauer larval formation allowing the worm to survive periods of food scarcity. *age-1* and *daf-2* seem to suppress the activity of the downstream target *daf-16*, a forkhead transcription factor[51]. Hence, loss of function of either of these upstream regulators enhances *daf-16* function and leads to increased life span. Importantly, loss-of-function mutations in *daf-16* not only prevent longevity conferred by the *age-1* and *daf-2* mutations, but also abolish stress resistance[52], thus strengthening the intimate link between longevity and stress responsiveness associated with this pathway. What remains to be determined is how the dauer mutations influence stress responsiveness and longevity. Identification of the transcriptional targets of daf-16 should provide insight into this process. Another *C. elegans* gene whose mutation confers both increased longevity and enhanced stress responsiveness is *clk-1* (ref. 53). *clk-1* encodes a mitochondrial protein homologous to a yeast protein involved in the synthesis of coenzyme Q, an electron carrier required for respiration (Fig. 2). Although its biological function in the nematode is unclear, *clk-1* mutants are believed to enhance life span by reducing the rate of metabolism, which in turn might lead to slower accumulation of damage resulting from metabolic by-products such as ROS. Consistent with this hypothesis, overexpression of *clk-1* leads to a reduction in life span[54].

Gene mutations resulting in reduced life span in *C. elegans* have also been described. Although a life-shortening phenotype is generally considered to be less reliable than a life-lengthening trait for the assessment of ageing-associated genes, two such mutations, *mev-1* and *ctl-1*, are worth special mention as they seem to be linked to oxidative stress. *mev-1* encodes a subunit of the enzyme succinate dehydrogenase cytochrome *b*, a component of complex II of the mitochondrial electron transport chain[55]. These animals have compromised mitochondrial function, reduced cytoplasmic SOD activity and show hypersensitivity to oxygen. *ctl-1* encodes a unique catalase localized in the cytosol[56]. It is likely to contribute to the cell's antioxidant defences, but *ctl-1* mutants have not been examined directly for responsiveness to oxidative stress. Interestingly, loss of *ctl-1* function also eliminates longevity conferred by both the dauer pathway mutants and *clk-1*. Additional evidence for a link between longevity and oxidative stress resistance in the nematode has recently been obtained using a pharmacological approach to boost antioxidant defences[57]. Treatment of wild-type *C. elegans* with synthetic SOD/catalase mimetics was shown to extend the mean life span by 44%. Moreover, the agent was also effective in restoring normal life span to *mev-1* mutants.

A similar link between longevity and stress resistance also exists in *Drosophila*. Various strains of flies selected for extended life span display increased resistance to oxidative stress that in some cases is also correlated with enhanced activity of antioxidant enzymes[58,59]. In addition, at least one gene, *methuselah* (*mth*), has been identified whose mutation not only enhances longevity but also increases resistance to heat stress and paraquat (an intracellular ROS generator)[60]. Further support for the relationship between stress tolerance and life span in *Drosophila* has been obtained using a transgenic approach to overexpress antioxidant genes. Overexpression of glutathione reductase, an enzyme involved in the generation of reduced glutathione, increases the resistance of flies exposed to hyperoxic conditions and extends life span under such conditions, although it does not affect life span of flies maintained in normal air[61]. Several studies have shown that elevated expression of CuZn-SOD alone or in combination with catalase likewise results in enhanced stress tolerance and increased life span[62–64]. However, other studies have reported little or no extension in longevity or enhanced stress

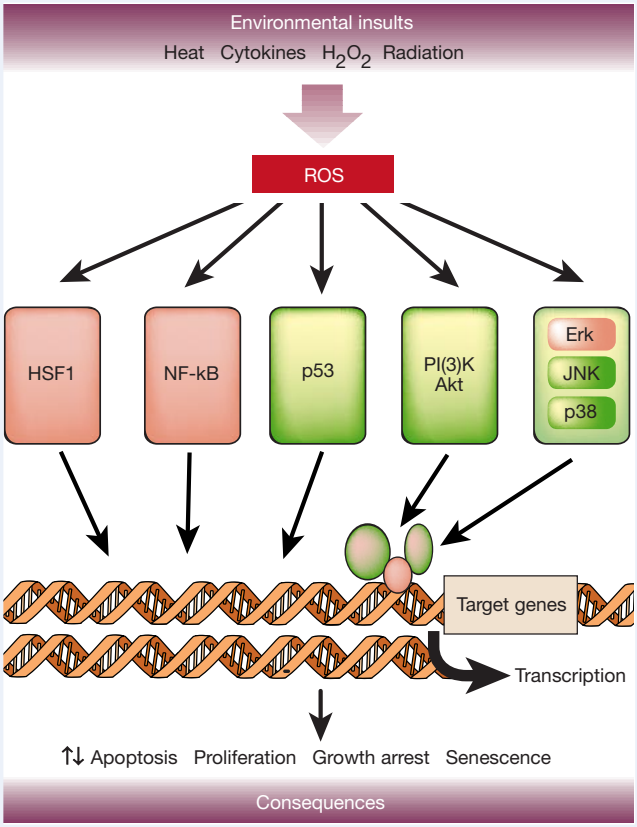

Figure 3 Major signalling pathways activated in response to oxidative stress. ROS can originate outside the cell, or may be generated intracellularly in response to external stimuli. Heat-shock transcription factor 1 (HSF1), NF-κB and p53 are themselves transcription factors, while the PI(3)K/Akt and MAPK pathways regulate transcription factors through phosphorylation. The degree to which a given pathway is activated is highly dependent on the nature and duration of the stress, as well as the cell type. The consequences of the response vary widely, with the ultimate outcome being dependent on the balance between these stress-activated pathways. HSF1 is responsible for activation of the heat-shock response. Factors depicted in pink represent those pathways whose activities are altered with ageing.

Box 1
Oxidants and diseases of ageing

Although maximum life span is the most relevant and defined endpoint with regard to ageing, it seems likely that in large multicellular organisms ageing need not proceed uniformly. This concept of non-uniform or focal ageing, or what has been termed segmental progeria by others[83], may be particularly important in a variety of age-related human diseases. Certainly the incidence of a host of diseases, including both cardiovascular and neurodegenerative disorders, increases exponentially with age. The basis for this steep rise in disease incidence is unexplained, but one possibility is that such diseases may share common mechanisms with ageing. In this regard, increasing evidence indicates that reactive oxygen species (ROS) may participate in the pathogenesis of these diseases. In support of this notion is the experimental evidence that the vessel wall of patients with atherosclerotic risk factors, but no overt disease, is characterized by a significant increase in vascular ROS production[86]. In addition, many ophthalmological and neurodegenerative diseases seem to be mediated, at least in part, by oxidative stress.

One common feature to many of these conditions is the recruitment of inflammatory cells. These cells contribute to oxidative stress in large part because they contain the potent NADPH oxidase system. Once activated, the NADPH oxidase complex, which is present in neutrophils, macrophages, microglia and vascular cells, produces large amounts of superoxide. In both the brain and the vessel wall the interaction of superoxide with endogenously generated nitric oxide can lead to the formation of peroxynitrite and other damaging radical species. Although the relative contribution of these various radical species is unknown, the link between inflammation and ROS seems to provide a useful framework for understanding disease progression.

Although laboratory data clearly suggest a role for oxidants in the pathogenesis of many age-related diseases, the clinical use of antioxidant therapy has been at best equivocal. Indeed, most of the large randomized trials of antioxidant vitamins such as vitamin A and vitamin E have shown, at best, marginal results and occasionally, potentially harmful outcomes (see Table below). Nonetheless, antioxidant therapy is in its infancy and issues of dosing and duration remain largely unexplored. It therefore is interesting to speculate that, in contrast to the relatively rare progerias that are characterized by accelerated global ageing, the clinical manifestations of these more common diseases, namely an atherosclerotic plaque or an Alzheimer tangle, may represent the fingerprint of accelerated but focal ageing.

Box 1 Table **Oxidants, antioxidants and diseases of ageing**

Disease system	Laboratory/animal studies	Clinical data
Cardiovascular	Pre-atherosclerotic blood vessels have increased levels of ROS[86]	PHS I: no overall benefit of beta-carotene on CVD? Benefit in high-risk subgroup[99]
	Vitamin E protects against development of atherosclerosis[87]	CHAOS trail: vitamin E reduces rate of non-fatal myocardial infarct[100]
	Disruption of SOD leads to heart failure[88,89] and overexpression protects against injury[90]	ATBC study: no overall benefit on CVD rate with vitamin E or beta-carotene? [1] Increase in CVD deaths with beta-carotene[10]
Ophthalmological	Offspring of pregnant mice depleted of glutathione develop cataracts[91]	PHS I: non-significant reduction in cataracts and macular degeneration with vitamin E and multivitamins[102]
	Retinal pigments produce ROS after light exposure[92]	NHS: carotenoids intake may decrease risk of cataracts[103]
	Retinal degeneration in primates with vitamin A or E deficiencies[93]	
Neurological	Mutations in SOD1 result in human ALS[94] and transgenic animal models rescued by antioxidants[95,96]	Vitamin E not protective in early Parkinson's disease[104]
	NMDA-receptor stimulation produces superoxide[97]	Vitamin E beneficial in Alzheimer's disease[105]
	Defects in the function of complex 1 seen in Parkinson's disease[98]	*N*-acetylcysteine does not effect survival in ALS[106]

The references cited above should be viewed as only representative examples derived from a much larger, relevant body of literature, which owing to space constraints cannot be fully presented. Acronyms and abbreviations: PHS I, Physicians' Health Study I; CHAOS, Cambridge Heart Antioxidant Study; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; NHS, Nurses' Health Study; CVD, cardiovascular disease; ALS, amyotrophic lateral sclerosis; NMDA, *N*-methyl-D-aspartate glutamate receptors.

resistance with overexpression of CuZn-SOD (refs 65, 66), and the basis for these discrepancies is presently unclear.

The effect of elevated SOD expression on stress resistance and life span has also been examined in mice. Although several reports have produced evidence to indicate that elevated CuZn-SOD levels afford protection against acute oxidative stress, a recent study showed that such acute protection did not correlate with increased longevity[67]. However, another study found an unexpected correlation between life span and control of the oxidative stress response: targeted mutation of the *shc* gene locus, which caused ablation of a splice variant of relative molecular mass 66,000 ($p66^{shc}$), resulted in selective resistance to oxidative stress and extended life span[68]. $p66^{shc}$ is one of three splice variants derived from the *shc* locus. $p52^{shc}$ and $p46^{shc}$ are important adaptor proteins that are regulated by tyrosine phosphorylation and participate in growth-factor- and stress-induced ERK activation. $p66^{shc}$ also undergoes tyrosine phosphorylation in response to extracellular signals, but its role in regulating ERK activation is less clear. However, the $p66^{shc}$ variant is unique in that it also becomes phosphorylated on serine residues in response to certain extracellular signals and, in the case of oxidative stress, this leads to apoptosis. Serine phosphorylation of $p66^{shc}$ is also stimulated by insulin[69]. This observation could provide another link to stress resistance and longevity, as an insulin-like pathway is clearly involved in regulating longevity and stress resistance in *C. elegans* (see above). Although better understanding of the mechanisms involved in $p66^{shc}$-mediated apoptosis and its importance to ageing await further investigation, these studies provide the first evidence for a genetic link between longevity and oxidative stress resistance in mammals.

Caloric restriction and oxidative stress

Limiting food intake, or caloric restriction, has been shown to extend life span in a wide range of species and in rodents it also slows the progression of a variety of age-associated diseases[70]. Although several theories have been advanced over the years to explain the anti-ageing effects of caloric restriction, one favoured hypothesis proposes that it acts by decreasing oxidative stress[71]. In support of this hypothesis, the rate of oxidant generation of mitochondria from calorically restricted mice is significantly lower than from their ad libitum-fed counterparts, and caloric restriction reduces the age-associated accumulation of oxidatively damaged proteins, lipids and DNA[70]. Caloric restriction also prevents many of the changes in gene expression and transcription-factor activity that normally occur with ageing,

including basal elevations in expression of heat-shock proteins[40,41] and attenuation of stress-induced Hsp70 expression[72]. Finally, caloric restriction increases the ability of rodents to withstand a wide range of physiological stresses, improves thermotolerance and reduces heat-induced cellular damage in aged rats[73].

Therapeutic strategies against ageing

Understandably, there is tremendous public interest in the development of anti-ageing therapies. If one accepts the evidence that oxidative stress has a significant role in ageing processes, and that the ability to resist or prevent oxidative stress is a key determinant of longevity, then it is likely that strategies aimed either at reducing the oxidative burden or boosting host defence mechanisms involved in coping with the damage would have significant anti-ageing effects (see Box 1). Two key issues will then arise. First, can effective therapeutics be designed to combat oxidant damage? Second, would such approaches be effective in retarding ageing and/or preventing diseases associated with ageing? It is already known that caloric restriction fulfils both of these criteria in lower organisms, and experiments in non-human primates have produced encouraging results to suggest that reducing food intake might also delay ageing in humans[74]. However, the possible application of caloric restriction to the human population would surely face insurmountable ethical and practical difficulties that would prevent its use. On the other hand, better understanding of the specific mechanisms responsible for the anti-ageing influences of caloric restriction could lead to the development of pharmacological agents (that is, caloric restriction mimetics) that would be effective in slowing ageing or delaying the onset of age-associated diseases.

Enhancement of antioxidant defences through dietary supplementation would seem to provide a more reasonable and practical approach to reduce the level of oxidative stress and there is a wealth of evidence to support the effectiveness of such a strategy *in vitro*. However, although some success has been achieved using antioxidant therapy in mammalian models of disorders induced by oxidative stress, so far these strategies have had little or no effect in enhancing longevity[75,76]. Successful application of this approach will probably require much greater understanding of the pharmacological properties of many of the agents being used, including their rates of absorption, tissue distribution, metabolism and the microenvironment in which they must act. Indeed, the problem is clearly more complicated than simply adding a pharmacological agent, as most free-radical scavengers act in oxidation-reduction reactions that are reversible, and some, such as ascorbate, can act both as antioxidants and pro-oxidants, depending on the conditions[77]. In addition, given the role of ROS as mediators of normal signalling processes, determination of the optimal dosage of supplements may require fine tuning to avoid overshooting the desired effects to the point of perturbing the delicate redox balance required for the maintenance of normal cell functions (Fig. 1). However, the development of new synthetic compounds that act as mimetics of SOD and catalase has offered an alternative approach with some promise[78,79]. Use of such compounds in mouse models has been effective in attenuating oxidative stress-associated disease processes, and leads to extension of longevity in *C. elegans*[57].

Finally, consideration should be given to strategies to boost the host defence mechanisms that are known to be activated in response to oxidative stress. So far, the best mechanism for boosting such responses seems to be stress itself. That is, a sublethal or conditioning stress can lead to enhanced survival and reduced tissue damage following a subsequent, more severe stress. This concept, termed hormesis[39,70,71], is in many ways the physiological equivalent of the philosophical notion that 'what won't kill you, will make you strong'. Although the basis for this stress tolerance phenomenon is poorly understood, elevations in heat-shock proteins may be one important factor. Interestingly, such conditioning stress can also lead to life extension in both *C. elegans*[80,81] and *Drosophila*[82]. In humans as well as lower mammals, exercise is believed to have many anti-ageing benefits. Because even in conditioned athletes exercise results in acute stress, it is possible that some of its benefits are derived through such a stress-tolerance mechanism.

Conclusions

In summary, although ageing is likely to be a multifactorial process, there is now significant evidence implicating the generation of ROS and the corresponding response to oxidative stress as key factors in determining longevity. Much of the early evidence was correlative, such as the relationship between metabolic rate and life span, and the observed increase in oxidative damage as a function of age. However, the more recent identification of longevity-influencing genes, first in lower organisms and subsequently in mammals, significantly strengthens the mechanistic connection between oxidants, stress and ageing. Many questions remain including how these various longevity-associated genes actually function to influence stress resistance and ageing, and how applicable they are to human ageing. If oxidative stress does indeed contribute to ageing in the manner described, do ROS act purely as random, destructive agents or — as we have intimated in this review — as regulators of discrete pathways linked to stress responsiveness and ageing? Similarly, it remains unclear whether it is the absolute level of oxidative stress encountered or the subsequent appropriate or inappropriate response to oxidative stress, or perhaps some combination of both, that ultimately determines life expectancy. Finally, and very importantly, in what fashion does oxidative stress intersect, if at all, with other proposed determinants of ageing such as telomere length and telomerase activity, or gene products such as the Werner's helicase that are linked to accelerated ageing syndromes? Nearly a century after the rate-of-living hypothesis was first proposed, the answers to these and related questions remain largely unknown. However, given the rapid rate at which our knowledge in this area has increased over the past few years, it is likely that answers to many of these questions will be forthcoming within this first decade of the new century. These answers will undoubtedly help determine whether ROS are merely peripheral targets that correlate with longevity, or instead, are finally established as a central regulator of human ageing. □

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