



HHS Public Access

Author manuscript

Free Radic Biol Med. Author manuscript; available in PMC 2019 August 20.

Published in final edited form as:

Free Radic Biol Med. 2018 August 20; 124: 420–430. doi:10.1016/j.freeradbiomed.2018.06.016.

ADAPTIVE HOMEOSTASIS AND THE FREE RADICAL THEORY OF AGEING

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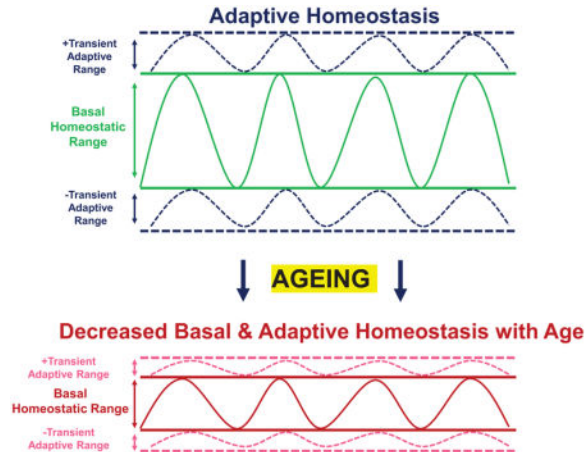
Abstract

The Free Radical Theory of Ageing, was first proposed by Denham Harman in the mid-1950's, based largely on work conducted by Rebeca Gerschman and Daniel Gilbert. At its core, the Free Radical Theory of Ageing posits that free radical and related oxidants, from the environment and internal metabolism, cause damage to cellular constituents that, over time, result in an accumulation of structural and functional problems. Several variations on the original concept have been advanced over the past six decades, including the suggestion of a central role for mitochondria-derived reactive species, and the proposal of an age-related decline in the effectiveness of protein, lipid, and DNA repair systems. Such innovations have helped the Free Radical Theory of Aging to achieve widespread popularity. Nevertheless, an ever-growing number of apparent 'exceptions' to the Theory have seriously undermined its acceptance. In part, we suggest, this has resulted from a rather simplistic experimental approach of knocking-out, knocking-down, knocking-in, or overexpressing antioxidant-related genes to determine effects on lifespan. In some cases such experiments have yielded results that appear to support the Free Radical Theory of Aging, but there are just as many published papers that appear to contradict the Theory. We suggest that free radicals and related oxidants are but one subset of stressors with which all life forms must cope over their lifespans. Adaptive Homeostasis is the mechanism by which organisms dynamically expand or contract the homeostatic range of stress defense and repair systems, employing a veritable armory of signal transduction pathways (such as the Keap1-Nrf2 system) to generate a complex profile of inducible and enzymatic protection that best fits the particular need. Viewed as a component of Adaptive Homeostasis, the Free Radical Theory of Aging appears both viable and robust.

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Graphical Abstract



Keywords

Ageing; Stress Adaptation; Signal transduction pathways; Nrf2; Proteasome; Oxidative stress; Protein oxidation and aggregation

INTRODUCTION

Ageing is a universal leveler and a process that has long fascinated humankind. One of the earliest records of our desire to slow or reverse the ageing process may be found in the writings of the 5th century Greek historian Herodotus and, since then, we have continually sought the elusive ‘fountain of youth.’

Indeed, the scientific community has generated over 300 theories in an attempt to explain the driving forces behind ageing [1], but none have, so far, been proven to be universally applicable. Rather, the multitude of extant theories highlights the complexity of the ageing process. One of the most popular postulates is the Free Radical Theory of Ageing, which has become one of the leading explanations to describe the ageing process at the molecular level.

Since its conception in 1956, the Free Radical Theory of Ageing, first hypothesized by Denham Harman [2], has greatly influenced the direction of ageing research for the past half century. Harman’s Free Radical theory is actually based on the original and independent observational work of Rebeca Gershman (from the University of Buenos Aires in Argentina) and Daniel Gilbert (from the University of Rochester) [3]. Gershman and Gilbert first identified the similar characteristics between reactive species produced during cellular respiration and those generated by irradiation: namely, the generation of free radicals, such as the hydroxyl ($\bullet\text{OH}$) and superoxide ($\text{O}_2^{\bullet-}$) radicals and the less reactive non-radical oxygen species, hydrogen peroxide (H_2O_2) [3].

Yet, at the time of Gershman and Gilbert’s observation, little was understood about the specific sites of free radical generation within the cell. We now know that the mitochondria account for one of the primary sources of intracellular reactive oxygen species (~1–5%), due

to electron leakage primarily resulting from the electron transport chain [4, 5]. One of the main byproducts is the formation of the highly reactive hydroxyl radical, which can either immediately react and damage neighboring mitochondrial proteins, DNA, and lipids or be converted to the less reactive, but longer-lived superoxide radical [6]. In turn, the superoxide radical can either react with molecules or undergo dismutation to hydrogen peroxide, which is highly dispersive within the cell and is the least reactive mitochondria-generated molecule [7].

Reactive species also arise from peroxisomes, which are the site of fatty acid oxidation. Hydrogen peroxide is the major free radical produced by acyl CoA oxidase in the metabolic breakdown of fatty acids [8], with the majority removed by peroxisome catalase and glutathione peroxidase. However, a small percentage can either leak into the cytosol or form the hydroxyl radical, due to Fenton chemistry. As well, superoxide, a byproduct of xanthine breakdown by xanthine oxidase [9], can either undergo dismutation by Copper-Zinc

Superoxide Dismutase (CuZn SOD), further adding to the peroxisome pool of hydrogen peroxide or react with the nitric oxide radical (a byproduct of peroxisome-mediated amino acid metabolism) to form peroxynitrite [10, 11]. As a result, cellular metabolism was considered the primary culprit for intercellular generation of so-called 'harmful' free radicals.

Indeed, Harman's theory postulated that if these highly unstable and reactive molecules are not removed or neutralized, they could damage proteins, lipids, and DNA either directly or indirectly (via Fenton chemistry) [2, 12]. Thus, Harman first proposed, that ageing occurred because of:

“.....deleterious side attacks of free radicals (which are normally produced in the course of cellular metabolism) on cell constituents and on the connective tissues” [2].

Harman's theory sparked the idea that complete removal of so-called 'harmful' molecules would mitigate cellular damage and, consequently, slow the overall ageing process. Indeed, studies have found that chronic removal of some antioxidant enzymes, such as cytosolic or mitochondrial superoxide dismutases negatively impacts the health-span and/or the lifespan in multiple model organisms [13–15]. However, it should be noted that the reverse is not always true, as multiple studies have shown that chronic over-expression of several antioxidant and detoxification enzymes can actually have little or no impact (and in some circumstances can even be detrimental) [16–20]. Over the years, people have tried to 'adapt' the Free radical

Theory of Ageing to accommodate various new, sometimes apparently contradictory findings, including a central role for mitochondria-derived reactive species [6, 21] and age-dependent dysfunction of protein, lipid, and DNA repair systems [22]. Though novel for its time, the Free Radical Theory of Ageing has since come under the scientific 'firing squad,' as multiple studies have concluded that it is not a universal phenomenon. For example, naked mole-rats live for some 32 years, yet levels of the reactive oxygen species they experience appear similar to those encountered by their short-lived cousin, the mouse [23].

Similarly, quails and parrots both produce similar levels of reactive oxygen species, yet parrots live five times longer than do quails [24]. Moreover, long-lived species [25, 26] and centenarians [27] show higher amounts of oxidative damage, measured by increased protein carbonyls, when compared to the average living animal or human, which is contradictory to Harman's theory.

It must be said that our understanding of the chemical and biological implications of free radicals has evolved significantly since 1956. The outdated binary (good or bad) belief, originally proposed by Harman, that free radicals and reactive oxygen species can *only* be deleterious molecules, while antioxidants and free radical scavengers must *always* be beneficial molecules, may no longer be sufficient. Indeed, this binary relationship was first tested by Linus Pauling, himself a strong proponent of very high daily-doses of ascorbic acid (Vitamin C) as an anti-ageing and disease intervention [28]. Nevertheless, Pauling's findings (and those of his followers), are highly controversial and do not demonstrate a definitive benefit [29–31]. Thus, simplistic binary beliefs about free radicals may no longer be an appropriate dogma.

Instead, growing evidence suggests it is the dynamic nature of production and decay, and thus the transient levels of various reactive oxygen and nitrogen species, which can tilt them as either beneficial or harmful. Short-term low concentrations of free radicals and reactive oxygen species make them advantageous (signaling), whereas too much and they become deleterious. Studies in yeast [32] and cell culture [33–40] attest to the beneficial non-damaging, signaling response elicited by transient exposure to H₂O₂, as low (μM) amounts rather than inhibiting growth (a sign of damage), actually stimulates proliferation, suggesting low amounts of free radicals can be beneficial. Moreover, pretreatment with short-term signaling amounts of a variety of free-radical generators, rather than increasing cellular susceptibility to damage, stimulates transient induction of cellular protective enzymes, indicating their role in elucidating a nuclear response [41–43]. As well evidence extends beyond cell culture models into model organisms, which show similar short-term exposure to low-amounts of free radicals and free-radical generators as protective rather than detrimental [18, 42, 44–47], with evidence suggesting early-life exposure even capable of promoting longevity [48].

Antioxidant and cellular defense enzymes also act as conduits for free radical signaling. Under homeostatic conditions, peroxiredoxins are predicted to breakdown the majority of mitochondrial and cytosolic-derived H₂O₂: at homeostatic signaling concentrations, peroxiredoxins are capable of continual H₂O₂ elimination [49, 50]. However, upon elevated H₂O₂ concentration, leads to peroxiredoxin inactivation and H₂O₂ accumulation. This triggers a cascade of cellular signaling events, beginning with the oxidation of target proteins and subsequent opening of the cellular defensive response [51]. One of considerable importance being the Nrf2 pathway, wherein modification of the thiol groups on Keap1, initiates Nrf2 nuclear translocation and subsequent activation of the stress protective pathway [52]. Thus, oxidant levels are highly responsible for initiating cellular signaling responses.

Conversely, more is not always better, as too much antioxidants can eliminate free-radical mediated cellular-signaling [51, 53]. Growing evidence shows free radicals are not only important for cellular homeostasis, but loss in their homeostatic balance may be an underlying cause for age-associated diseases, and ageing itself [54–56].

THE FREE RADICAL THEORY OF AGEING

Is More Always Better – and Less Always Worse?

The main premise of the Free Radical Theory argues that ageing is a result of cellular damage accumulation due to free radical attack. Based on this statement, it would appear that at least two outcome predictions should hold true. The first being that if antioxidant and stress-related cellular defenses are removed, then an ‘ageing’ phenotype should appear. Conversely, overexpression of these same enzymes should result in suppression of the ‘ageing’ phenotype and, perhaps even promote lifespan.

Much effort has been invested in addressing the validity of the first predicted outcome, primarily by the chronic knockdown of antioxidant or stress-responsive enzymes. Initial work in baker’s yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*), found that elimination of either cytosolic Cu-Zn-Superoxide Dismutase (SOD1) or the mitochondrial Mn-SOD variant (SOD2), the primary enzymes responsible for the breakdown of the superoxide radical, resulted in decreased survival during stationary phase [57]. Yeast survival was even further diminished (presumably a synergistic decline) observed in the SOD1/SOD2 double mutant [57]. Similar outcomes were noted for some other stress responsive enzymes, including the yeast homologues for the 20S Proteasome, Proteinase yscE [58], the ATPase subunit of the 26S Proteasome [59] and the mitochondrial Lon protease, Pim1 [60, 61], all of which are necessary for the removal of oxidized and/or ubiquitin-tagged proteins. Chronic knockdown of the hydrogen peroxide-detoxifying enzyme, catalase, however, actually improved chronological aging in yeast, suggesting that loss of antioxidant enzymes is not universally detrimental, and providing evidence against the Free Radical Theory of Ageing [62].

Parallel work in *Drosophila melanogaster* (*D. melanogaster*), or more commonly known as fruit-flies, found that the removal of major antioxidant enzymes, such as the mitochondrial SOD2 [63–65], resulted in shortened lifespan. Similar findings were noted for chronic knockdown of cytosolic SOD1 [66, 67]. Removal of stress-responsive enzymes, such as the 20S proteasome and the mitochondrial Lon protease, also showed decreased survival [18, 45, 68]. Similarly, inactivation of the 20S Proteasome led to shortened lifespan [69]. Conversely, mutants lacking full or partial catalase activity showed no change in survivorship [70].

Studies in the nematode worm, *Caenorhabditis elegans* (*C. elegans*), represent an even greater experimental contradiction of the first component of the Free Radical Theory of Ageing. Unlike studies in yeast and fruit-flies, removal of certain stress responsive and antioxidant enzymes had either no impact or, in some instances, were actually beneficial. For example, individual removal of the five superoxide dismutase (SOD) genes caused no change in *C. elegans* lifespan, and mutants lacking two or three superoxide dismutase genes lived as long as the wild type [71]. Moreover, worms deficient in individual superoxide

dismutase genes showed increased mRNA levels in the remaining *sod* genes [72]. Removal of glutathione transferase did not appear to impact survival at all [73]. Confusingly, removal of the mitochondrial Lon protease resulted in increased lifespan [74], whereas, removal of 20S Proteasome or 26S Proteasome subunits, or their transcriptional activator Nrf2, consistently resulted in shortened lifespan [75–77], even though both Lon and the Proteasome play important roles in clearance of (oxidatively) damaged proteins. In contrast, removal of nuclear-encoded proteins involved in mitochondrial electron transport and oxidative phosphorylation (a primary source of mitochondria-derived O_2^- and H_2O_2) has been linked with increased longevity, which is actually in line with the first predicted outcome of the Free Radical Theory of Aging [78]. These highly conflicting and contradictory outcomes in the worm either highlight a species-specific anomaly, which would certainly challenge the universality of the Free Radical Theory of Ageing, or may hint at even deeper potential flaws in the theory.

Interestingly, as studies progressed into mice, survival outcomes tended to align more closely to those in the nematode worm. Indeed, mice lacking crucial antioxidant and stress-responsive enzymes showed inconsistencies in lifespan following their removal. The levels of several antioxidant gene products have been diminished or eliminated by knockdown or knockout techniques to generate a variety experimental mice, including *sod2*^{+/-} [79], *gpx4*^{+/-} [80], *gpx1*^{+/-} [13], *msrA*^{-/-} [81], *sod1*^{-/-} [82], and *trx2*^{+/-} [82] animals. Of these six genetically modified mice strains, only the *sod1*^{-/-} animal, deficient in cytosolic Cu-Zn SOD, exhibited decreased survival. Importantly, even when multiple antioxidant ‘redundancy’ pathways were eliminated, no changes in survival were observed [13, 82]. It should, however, be noted that full knockdown of *gpx4* and *sod2* were identified as embryonically lethal [83, 84]. It should also be remembered that some of these studies were executed with mixed populations of mice, thus any sex-specific differences may not be evident.

Given the results reviewed above, it should be of little surprise that chronic overexpression is highly organism-dependent. Nevertheless, one can certainly point to quite a large number of studies in which overexpression of antioxidant enzymes resulted in positive effects on survival and/or lifespan. In *S. cerevisiae*, for example, chronic upregulation of antioxidant and stress-responsive enzymes are largely beneficial. In the case of the proteasome, increased expression leads to enhanced activity and increased replicative lifespan [85], with a similar trend evident upon overexpression of SOD1 and SOD2 [86, 87]. Additionally, chronic upregulation of other detoxification enzymes, including catalase, has provided evidence for improved survival [88, 89]. Similar findings are also evident in mammalian cell culture studies [90–92]. Furthermore, overexpression of cytosolic and mitochondrial forms of SOD [93–96] increased lifespan in *D. melanogaster*. In one finding, highly active SOD variants in *D. melanogaster* neurons were associated with exceptional longevity [97]. Enhanced expression of the antioxidant enzymes, glutathione-S-transferase [98], glutathione reductase [99], SOD1 [67] and SOD2 [100], all improved survival in the nematode worm. Finally, overexpression of the beta 5 catalytic subunit of the 20S proteasome resulted in increased survival [101], and indirect proteasome upregulation (achieved through the suppression of germline development) also led to enhanced longevity [102].

Despite the positive results detailed above, little benefit of increased antioxidant gene expression has been found in higher organisms. For example, of the vast majority of lifespan studies completed using transgenic mouse strains, overexpressing antioxidant or oxidant-repair genes, only a small percentage have actually proven beneficial [103, 104]. Indeed, overexpression of the antioxidant genes SOD1, SOD2, GPx4, catalase [19, 82, 105] either individually, or as synergistic pairs (SOD1 + SOD2, or SOD1 + catalase) [19, 82] failed to increase lifespan and, in some instances, actually shortened life [106, 107]. In a similar manner, the forced over-expression of stress-responsive enzymes, rather than being beneficial has often been found to be detrimental. For example, mice transgenic for mitochondrial Lon protease (Lonp1^{+/+}) showed increased tumorigenesis [108].

These lack-luster findings and inconsistencies both across and within species, have led to increased interest in the role of upstream targets. One of the most notable of these is the nuclear factor erythroid 2-related factor 2 (Nrf2), which is a major transcriptional regulator of numerous Phase II detoxification and stress-responsive enzymes [52]. Due to its important role as a master transcriptional regulator, much interest has centered upon understanding the impact of Nrf2 levels upon survival. Yet, even these findings are inconsistent across models. For example, decreased survival has been reported upon Nrf2 suppression in *C. elegans* [109] and *D. melanogaster* [110] and overexpression is beneficial to lifespan [45, 111]. Yet, in a mammalian mouse model, Nrf2 knockout resulted in no change in lifespan, but increased sensitivity to oxidative stress [112] and tumorigenesis [113]. Whereas knockout of the Nrf2 cytosolic inhibitor, Keap1, results in death after 20 days [114]. Overall, inconsistencies in Nrf2-mediated survival suggest a more nuanced mechanism is at play.

On aggregate, these outcomes suggest that either the binary historical approach in the interpretation of the Free Radical Theory of Ageing is incorrect, with current means of addressing its validity are oversimplified, or that the theory itself may be flawed. We would suggest instead, that these outcomes serve to highlight the adaptability and pathway redundancies that exist, enabling organisms to cope (and even live a normal lifespan), despite significant negative or positive perturbations of specific detoxification or stress-responsive enzymes. Therefore it brings into question, whether weaknesses become evident when the system is perturbed or no longer within its homeostatic range.

System Perturbation: Toppling the Biological House of Cards

A major flaw in addressing the validity of the Free Radical Theory of Ageing, is the widespread approach of forcing a biological system to adapt a new 'homeostatic' set-point (through the chronic increase or decrease of a target enzyme) and then assessing the outcome. In so doing, this model overlooks a fundamental component: no organism lives in isolation. Rather, cellular defenses (and their respective enzymes) are continually in a state of transient malleability. In turn, rapid and short-term modulation of enzyme levels, ensures an organism can cope with its environment. In contrast, chronic enzymatic expression prevents small and nuanced changes, which may be necessary for survival.

From this perspective, it should be of little surprise that limited lifespan benefit was found upon chronic expression of target enzymes. Moreover, enzyme manipulation was done in a state of relative non-perturbation (unless ageing itself, is viewed as a stress). The deeper nuances becomes evident when stress resistance was assessed in these studies. For clarity, we define stress resistance as the survivability of an organism when exposed to a high, semi-lethal amount of an oxidant or environmental treatment meant to promote cellular damage and/or death.

Indeed, evidence shows that the removal of enzymes critical for detoxification or protection against oxidative stress are more vulnerable when exposed to these semi-lethal concentrations. For example, fibroblasts from mice deficient in SOD1, though showed few abnormalities and normal lifespan, were highly sensitive to the superoxide generator, paraquat [115–117] and showed higher rates of tissue-specific DNA damage [118], accelerated protein oxidation [119], decreased Proteasome activity [120] and more recently proposed as a model for age-associated frailty [121]. A similar trend occurs in fibroblasts from glutathione peroxidase deficient-mice [122] and cells from mice deficient in mitochondrial SOD2^{-/+} [123], including an associated loss in mitochondrial aconitase activity and decreased Lon expression [124]. A synergistic effect in heightened oxidative stress sensitivity was noted in a double mutant mouse model of SOD^{+/-} and glutathione peroxidase^{-/-} [125]. Moreover, cells lacking stress-responsive enzymes show increased oxidative-stress sensitivity, including direct knockdown of the Lon protease [126] and the Proteasome [127].

Conversely, lack of an enzyme can result in activation of stress-responsive pathways. This is most prominent in studies manipulating the mitochondrial electron transport chain, one of the primary sources, argued by Harman, to contribute to cellular oxidative stress. Studies in mice lacking *surf*^{-/-} (an auxiliary protein in the aid of Complex IV), showed a compensatory activation of the unfolded protein response (UPR), leading to increased expression of UPR-associated proteins (including Nrf2, mitochondrial Lon protease, and heme-oxygenase-1) [128]. In a similar trend, mutations resulting in electron transport chain dysfunction, which should promote the generation of free radicals, rather than shortening the lifespan, triggered longevity in worms [78, 129], flies [130], and mice [131].

These differences in stress resistance, evident upon removal of antioxidant and/or stress-responsive enzymes, suggests that Harman's argument perhaps may not be flawed, rather our scientific approach may be too simplistic. For decades, we have asked the straightforward and simple question, if we increase or decrease enzyme "X" what is the outcome upon survival? Instead, we should be asking how does an organism adapt to stress and can it be enhanced in the absence or presence of an enzyme in a life-stage dependent manner.

ADAPTIVE HOMEOSTASIS AND AGEING

Although our understanding of the roles of free radicals and other reactive oxygen/nitrogen species has evolved to include important physiological functions in cellular signaling, our theories to adequately describe how such reactive species may affect the ageing process have not kept pace. To address this gap, we propose a new theory that builds on the Free Radical

Theory of Ageing, but also includes the dual roles of free radicals and other reactive oxygen/nitrogen species in cellular signaling. Importantly, our new theory is also much broader than the original Free Radical Theory of Ageing, in that it is also applicable to any and all agents or conditions that, at high concentrations, can cause cellular damage. Our new premise is based upon the concept of transient and reversible adaptation, or more aptly coined 'Adaptive Homeostasis,' which allows for short-term, transient changes in the homeostatic range of many protective and repair mechanisms. Adaptive Homeostasis has been defined as follows:

“The transient expansion or contraction of the homeostatic range in response to exposure to sub-toxic, non-damaging, signaling molecules or events, or the removal or cessation of such molecules or events.” [132].

Recently, much evidence has come to light to demonstrate both sex-specific [40, 133] and age-associated [54, 134–139] changes in the adaptive homeostatic response, which may more appropriately describe age-dependent changes and susceptibility to diseases. These findings all very clearly point to an age-determined decline in adaptive homeostasis that makes older cells, organisms, animals (and humans?) more sensitive to toxicants, other damaging agents, and harmful environments or conditions.

We, therefore, now formally propose an Adaptive Homeostasis Theory of Ageing, which we define as follows:

“Ageing involves a decline in the ability to activate or signal Adaptive Homeostasis to effect transient, short-term expansion or contraction of the homeostatic ranges of stress-protective systems. Since exposure to damaging, toxic, or stressful molecules and conditions still occurs, the net effect of diminished Adaptive Homeostasis is manifested as deterioration of biological structures and functions, with increasing accumulation of cellular and intracellular damage products in later life and eventual dysfunction, disease, senescence, and death.”

Our purpose in this paper is to demonstrate that the Adaptive Homeostasis Theory of Ageing helps to fill in inconsistencies in the Free Radical Theory of Ageing that have become evident over the past few decades, and to provide an alternative and broader theory that can encompass free radicals under its umbrella.

Assessment of the Adaptive Homeostatic Response

Prior work describing adaptation typically refers to the phenomenon as hormesis, wherein concentrations, typically toxic at high doses, become beneficial at lower concentrations [140–142]. Yet this beneficial viewpoint, does not match the original description posited by Southerland (1945), which argued, from a toxicology standpoint, the process in which a toxin or poison could cause sub-lethal damage, leading to an exaggerated repair response [143]. Or more aptly phrased by the 19th century German philosopher, Friedrich Nietzsche “that which does not kill us, makes us stronger.” This inaccurate, but prominent perception of hormesis, requires it to be distinguished from the adaptive homeostatic response, which involves non-damaging signaling amounts of an oxidant or environment to activate these same pathways [132].

Early studies in bacteria [144, 145] clearly demonstrated that *Escherichia coli* can undergo regulon-dependent protective adaptive changes in gene expression, with consequent transient increases in stress resistance, following exposure to very low levels of either $O_2^{\cdot-}$ (OxyR Regulon) or H_2O_2 (soxRS Regulon), and we (and others) have successfully repeated these results. We moved on to eukaryotic studies in *Saccharomyces cerevisiae* yeast and in mammalian cells (that don't possess regulons) which provided more detailed insights and eventually lead us to propose Adaptive Homeostasis.

Yeast, pretreated with low, non-damaging, amounts of H_2O_2 and subsequently challenged with a toxic amount of H_2O_2 , showed improved survival compared to yeast only subjected to the challenge dose of H_2O_2 . More importantly, pretreated yeast showed improved growth, even exceeding the density present in control colonies [32]. Later studies, using multiple types of mammalian cell lines (including human) found a consistent and similar phenomenon: pretreated cells showed increased survivorship compared to populations exposed only to an oxidant challenge [146–148]. In addition, cells were found to be capable of undergoing multiple cycles of adaptation, de-adaptation, and re-adaptation [32, 43, 146], suggesting the importance of the adaptive response in day-to-day cellular changes. Demonstration that cells can adapt, de-adapt, and re-adapt is important because it rules out the possibility that we are only selecting for a (possible) pre-existing sub-population of cells with innately high levels of oxidant protection.

The universality of the adaptive homeostatic response has also been tested. Indeed, evidence for its applicability is seen across multiple forms of cellular stress, including heat shock, osmotic stress, caloric restriction, hypoxia, cold shock, exercise training, and more [134, 149]. These findings summarize a unifying theme, wherein organisms rely upon a cadre of synergistic pathways to cope with environments that offer diverse stresses, often in combination. Assessing how an organism adapts to various stresses provides something of a 'fingerprint' of where it fits in its environmental niche. From such investigations it appears clear that the ability to rapidly respond to signal-inducing changes is crucial for long-term homeostasis and survival.

Adaptability of the Proteasome and Mitochondrial Lon Protease

The adaptive homeostatic response relies upon multiple stress-responsive pathways and enzymes to ensure appropriate clearance of cellular damage. The 20S Proteasome was one of the first enzymes identified as stress-responsive for protein clearance [34, 41, 150–157]. Although multiple forms of the Proteasome reside simultaneously within the cell, the 20S 'core' Proteasome is the predominant form under oxidative duress [158, 159]. The core 20S Proteasome is a barrel-shaped enzyme, comprised of two outer alpha- and two inner beta-rings, with the proteolytic activity contained within the beta rings (β_1 has caspase-like activity, β_2 has trypsin-like activity, and β_5 has chymotrypsin-like activity) [159]. Initial studies identified oxidized proteins as the preferred substrate of the 20S Proteasome [33, 34, 153, 154, 160, 161], with later work showing rapid elevation in proteolytic capacity upon oxidative stress [41, 43, 157].

Activation of the adaptive stress response leads to rapid disassembly of the 26S Proteasome by sequestering of the 19S oxidant-inactivated regulatory caps by HSP70 [162, 163]. HSP70

actually acts in conjunction with a partner enzyme, ECM29, first identified as a proteasome scaffolding protein in yeast [164, 165] and later found to have a conserved role in mammalian cells [166, 167]. This leads to an immediate increase in the cytosolic pool of 20S Proteasomes available to degrade oxidized proteins. HSP70 returns its 19S cargo to 20S Proteasomes (by an unknown mechanism) some 3–5 hours following the initiation of adaptive responses, thus ‘restoring’ 26S Proteasomes which are required over the following 13–15 hours for a fully effective adaptive response in mammalian cells. Importantly, also during the 3–5 hour period of 26S Proteasome disassembly, Nrf2 cannot be degraded by the ubiquitin-26S Proteasome system and Nrf2 levels rapidly rise. At the same time, Nrf2 nuclear translocation and transcriptional binding to Electrophile Responsive Elements (EpRE’s), also called Antioxidant Response Elements (ARE’s) within the upstream regulatory regions of many target genes [52] (including those encoding 20S Proteasome subunits) [41], leads to de novo synthesis and further elevation of the available 20S Proteasome pool [41, 168]. Collectively, these events ensure that the cell has both an immediate (minutes) and long-term (hours) source of available and functionally competent 20S Proteasome, with these responses appearing as an in-phase mediated process [169].

More recently, additional forms of the Proteasome have been found to be involved in the adaptive stress response. The immunoproteasome, first identified for its role in the innate immune response [170, 171], has since been coined the ‘Inducible’ Proteasome [172], as it too, is found to increase in response to oxidative stimuli [156, 157, 170, 173]. In addition, the so called PA28a β (or 11S) cytoplasmic regulator undergoes increased synthesis and binds to both 20S Proteasomes and Immunoproteasomes which improves their ability to degrade oxidized proteins. Together, these multiple pathways in the Proteasome adaptive response work synergistically to ensure cells and tissues are protected against oxidative insults.

Parallel studies have identified additional proteolytic stress responsive enzymes, one of the most notable being the mammalian mitochondrial LonP1 protease (encoded by the *Lon* gene). First identified in *E. coli*, as a heat-shock inducible protein (La) [174, 175], Lon was later found to be conserved from yeast (Pim1) [68] to mammals [176], as a characteristic six- (mammal) or seven-membered (yeast) monomeric ring, which degrades oxidized proteins in an ATP-dependent manner [177–179]. Initially discovered as a bacterial stress-inducible enzyme, a conserved role of Lon induction was identified in mammalian cell culture studies in response to multiple types of oxidative stress: including heat shock, hydrogen peroxide, hypoxia, endoplasmic reticulum stress, and serum starvation [37–39, 180–184]. Additional work has identified mammalian LonP1 as an indirect regulator of mitochondrial transcription, as it modulates mitochondrial transcription factor A (TFAM) turnover [108, 185, 186], and more recently its role in the Pink1/Parkin system, crucial in mitophagy [187]. Together, these findings indicate the dynamic role of Lon as highly responsive to the oxidative status and needs of the cell.

Can Model Organisms be Used to Explore and Better Understand Adaptive Homeostasis?

Our understanding of the eukaryotic adaptive stress response is primarily derived from mammalian cell culture studies. Yet, to explore the mechanism(s) and universality of the

adaptive homeostatic response in intact organisms, and its relevance to human beings, two caveats need to be met: first, one must be able to experimentally replicate the adaptive homeostatic response, seen in cells, in whole organisms; and second, one must be able to find model organisms in which the pathways for adaptive homeostasis are approachable, dissectible, and strongly related to mammalian/human pathways.

Initial studies used young (3 day old) worms and fruit-flies. Animals were either not pretreated or were pretreated with non-damaging, signal-transducing amounts of H₂O₂ (10–1000μM) for a short-duration, followed by an activation phase for the adaptive response, and finally application of a toxic (semi-lethal) dose of H₂O₂ (a 1.6mM solution in worms, and 4.4M in food on Kimwipes in fruit flies) and survival was measured. In both scenarios, pretreated organisms survived longer than those subjected only to the challenge dose [42]. Additionally, similar to the cell-culture studies discussed above, low signaling amounts of H₂O₂ stimulated increases in 20S Proteasome transcription/translation and activity in the nematode worm [42, 44] and fruit fly [42, 45]. Furthermore, induction of the 20S Proteasome was shown to be Nrf2-dependent [42, 44]. Finally, blocking induction of either the 20S Proteasome or the mitochondrial Lon protease blunted organismal adaptation and survival [18, 44, 45]. These findings indicate that Adaptive Homeostasis is conserved in higher organisms, and also provide us with a model for its assessment.

Assessment of the adaptive homeostatic response further shed light on the divergence in response between the self-fertilizing hermaphroditic worm and the heterogenic fruit-fly. Specifically, the fruit fly provided us with evidence for sex-specific differences in the adaptive response. Indeed, though earlier studies have found lifespan and stress resistant sex differences [188–190], much of the work in this field has been limited to one sex (male) [191]. In contrast, much of our own work on adaptive responses in mammalian cell-cultures are based upon female-derived cell lines [40]. In fruit-flies, 3 day old females were capable of adapting and inducing the 20S Proteasome in response to hydrogen peroxide signaling doses [45], which was found to hold true in multiple fly strains [192]. Additionally, the mitochondrial Lon protease not only showed a female-favored response upon hydrogen peroxide pretreatment, but was the first identification of different isoform patterns in females versus males [18]. Conversely, pretreatment with the superoxide generator, paraquat, found a male-dependent adaptive increase in the amount and proteolytic capacity of the 20S Proteasome [192] and mitochondrial Lon protease [18]. These findings indicate that not only are organisms capable of adaptation, but that the adaptive response is sex- and oxidant-dependent.

More recently, new evidence highlights the translational aspect of adaptive homeostasis in response to environmental factors. One that has gained increasing traction, is assessment of air pollution (specifically from vehicular-derived ‘smog’) and its impact upon human health. Yet, assessing the impact of low-level exposure upon the adaptive response is only in its infancy. To begin to address this gap, we utilized 6 month old C57BL/6 male mice, exposed to ambient air or vehicular-derived nanoparticles for a period of 10 weeks. Lung, liver, and the cerebellum were assessed for changes in Nrf2-responsive enzymes [47]. Interestingly, young males showed induction of Nrf2-phase II responsive genes, compared to controls, suggesting activation of the adaptive homeostatic response [47]. More recently, 6 month old

C57BL/6 female mice showed a similar trend, along with activation of the stress responsive enzymes, the mitochondrial Lon protease and the 20S proteasome [193]. Together these findings suggest the conserved role of the adaptive response in a mammalian animal and provide a model in which it may be further tested and explored.

Loss of the Adaptive Homeostatic Response with Age

Young organisms are characterized by the robustness of the adaptive homeostatic response: the reversible expansion or contraction of the homeostatic range, ensuring transient upregulation of protective pathways. However, if adaptation enables young organisms to cope in a dynamic environment, then its antithesis is ageing. Indeed, multiple studies have demonstrated, across multiple signal-transducing mediators of adaptive responses, an age-dependent attenuation [134]. Early work addressing this question showed that high-passage or senescent cells, were no longer capable of transiently activating the adaptive response. The concept of proliferative senescence, first demonstrated by Hayflick and colleagues, in which normal cells (in this case fibroblasts) have a finite number of divisions before they stop dividing [194], offered an excellent model for assessing senescence of the adaptive response. Indeed, high passage fibroblasts not only showed the classical decline in proliferation, and protein synthesis, but also decreased proteolytic capacity [35, 36, 126, 195–201]. Nor was this decline a result of lower amounts of the 20S Proteasome, as levels remained constant [35, 36, 202]. Since then, multiple studies using *in vitro* cellular senescence models, have revealed a similar decline in proteolytic capacity [202–204]. In addition, the decline in 20S proteolytic capacity holds true in primary human cell types from donors of increasing age [205, 206]. Moreover, fibroblasts from longer-lived species and centenarians show greater retention of 20S proteolytic capacity [207–209] and its regulator Nrf2 [210] into old-age, along with an associated elevation in the amount of the Immunoproteasome [208].

Direct assessment of protein clearance in tissues derived from aged animals reveals a similar decline in proteolytic capabilities. Specifically, human-derived tissues including heart [211], skin [212], and lymphocytes [213], exhibit a clear age-dependent loss of proteolytic capacity. A consistent ageing trend is also evident in tissues from mice [214, 215], rats [216, 217], and primates [208], and this finding is clearly accelerated in the disease states [218–224]. Additionally, studies in mice show a similar decline in Lon activity with age [124, 225], although much less work has focused upon age-associated proteolytic changes of the mitochondrial Lon protease. Together, these findings suggest a globally consistent and significant age-dependent attenuation of proteolytic activity and capacity.

From the evidence presented above, proponents of the Free Radical Theory of Ageing might well argue that the age-dependent loss in protein clearance is a *consequence* of damage accumulation, most likely arising from a lifetime's worth of free radical and oxidant exposure. While we do not dispute the importance of free radicals and oxidative stress in ageing processes, we would like to suggest that more nuanced mechanisms are involved, and to offer a somewhat different interpretation. We suggest that the age-dependent decline in proteolytic capacity results from decreasing ability to mount appropriate and sufficient adaptive cellular responses. During ageing, the rate and extent of protein oxidation may not

change, or it may actually increase, but cellular clearance of damaged proteins clearly declines [226, 227]. Importantly, however, this intracellular accumulation of aggregates of damaged (often cross-linked) proteins clearly is not a gradual process occurring over the course of a lifespan, but rather a phenomenon that characterizes the final third of life. This was very nicely demonstrated in a paper from Rodney Levine and Earl Stadtman who reviewed several studies, across multiple species, to show that intracellular oxidized protein aggregates do not accumulate during the first two thirds of lifespan but that such aggregates actively accumulate in an exponential fashion in the final third of life [228]. Interestingly, Harman also proposed exponential damage processes in ageing, but he suggested that they begin as early as the second third of life, after age 28, in persons living in Western societies [229].

As noted previously, one important factor in protein aggregation is the 20S Proteasome. Multiple findings suggest that it is not a lack of Proteasome, *per se*, but rather a diminished pool of functional Proteasome, which contributes to increased aggregation of oxidized proteins because they 'escape' degradation. In support of this interpretation, the Proteasome was identified as a major component of the 'insolubolome,' or insoluble protein aggregates [230]. Conversely, work in yeast, shows disaggregation of these protein clumps leads to increased proteolytic capacity [231].

Much of the work from our laboratory, and evidence found in the literature, has shown that ageing is associated with late-stage decline in activation of the adaptive response [134]. Unlike early passage cells, which demonstrate a clear response to adaptive signaling (*i.e.* Nrf2 transcriptional activation, stimulated proliferation, and increased proteolytic capacity), senescent cells show a clear failure or blockage of adaptive homeostasis. Indeed, pretreatment with normally stimulatory (non-damaging) low amounts of an oxidant in high passage cells, produces a sluggish (at best) induction of phase II enzymes and stress-responsive pathways [38, 232]. Moreover, this ageing phenotype could be mimicked if cells were exposed to Nrf2 inducers too frequently, or chronically [43]. Studies using primary human bronchial epithelial cells (HBE cells) from older individuals also exhibit an impaired Nrf2-mediated response compared with HBE cells from young people. HBE cells from older individuals, for example, are very poor at inducing Nrf2 target genes such as *GCLC*, *GCLM*, and *NQO1* following treatment with the known Nrf2 transcriptional activator sulforaphane, in comparison with HBE cells from young people [233].

Simpler, model organisms also show an age-related decline in the adaptive homeostatic response. Studies using aged *C. elegans* worms and *D. melanogaster* fruit flies, for example, have revealed loss of inducibility of the 20S Proteasome [44, 45] and the mitochondrial Lon protease with age [18]. In addition, aged worms and fruit flies, pretreated with a normally adaptive amount of an oxidant, show little or no increase in stress-resistance in comparison with their young counterparts who adapt very well [18, 44, 45], indicating an age-related loss in adaptation. The ability to modulate protein clearance in response to stress is also diminished in ageing. Whereas young animals, first pretreated with an adaptive amount of an oxidant and then subsequently challenged, are better able to mitigate accumulation of damaged proteins, aged pretreated animals exhibited no such increase in protein clearance [44, 45]. Additionally, studies using 21 month C57BL/6 male and female mice, exposed to

low amounts of smog-derived nanoparticulates, showed loss of induction of Phase-II responsive enzymes, in comparison with their young counterparts who adapted very well to the exposures [47, 193].

Another major age-dependent factor that should be noted is that, in all systems so far studied, ageing is associated with an increase in the basal levels of stress-responsive enzymes. This trend is consistent from cell culture to mammalian mouse models [134]. Nor is simply forcing the expression of stress-responsive systems able to restore the adaptive response in old animals, as chronic over-expression of the *C. elegans* Nrf2 homolog, Skn-1, failed to restore adaptive homeostasis [44]. Similarly, chronic knockdown in *D. melanogaster* of the Nrf2 cytosolic inhibitor, Keap1, showed improved stress resistance, but did not remediate the adaptive response [45]. When tested in mice, it was again found that baseline levels of Nrf2 increased with age, as did the levels of many Nrf2-inducible gene products (*e.g.* GCLC, GCLM, NQO1, HO1, Proteasome, and Lon) representing an upward contraction of the basal homeostatic range. In the same old animals, however, adaptive homeostasis declined to negligible levels in comparison with their young counterparts [47, 193].

In search of possible transcriptional players involved in the decline of Nrf2 responsiveness with ageing, we have found Bach1 and c-Myc as potentially viable candidates [47, 193]. Bach1 has been understood for some time to act as a competitive inhibitor of Nrf2-mediated signal transduction by forming heterodimers with MafK and MafG proteins that bind to the EpRE/ARE response elements. Docking of Bach1 sterically blocks the binding of Nrf2, leading to the repression of EpRE/ARE-mediated gene expression and induction [52, 234, 235]. In addition, c-Myc has been revealed as something of a non-traditional Nrf2 inhibitor, by means that have yet to be fully revealed, but which may well involve enhanced Nrf2 nuclear turnover [236]. Importantly, basal levels of both Bach1 and c-Myc have been found to increase with age in mice, and in human HBE cells, with concomitant declines in inducibility of Nrf2-regulated genes [47, 52, 193]. Thus, Bach1 and c-Myc may well represent viable targets for 'ageing intervention,' although one must always consider the possibility that these Nrf2 'inhibitors' increase with age for a positive reason, yet to be discerned.

Thus ageing may actually be considered as a quintessential example of the decline in adaptive homeostasis. Ageing results in a compression of the homeostatic range, with chronically high levels of expression of stress-resistant enzymes but, paradoxically, an actual decrease in stress protection. Furthermore, ageing results in a generalized loss of adaptive homeostasis, such that stress-protective systems and enzymes can no longer be appropriately induced by signaling pathways, such as Nrf2, that are highly effective protectants in young animals.

Conclusions

It would seem to us that, although frequently attacked and often badly wounded, the Free Radical Theory of Ageing continues to survive and even evolve. We would suggest, however, that simplistic interpretation or application of the Theory has led to confusion, and to unwarranted negative conclusions. Specifically, it now seems clear that one cannot

actually ‘test’ the Theory by simply intervening in one or two biochemical or genetic pathways and expecting that no other adaptive or counterbalancing factors, enzymes, or systems will compensate over the course of a lifetime.

We now realize that multiple protective systems against free radicals and oxidative stress are highly regulated and modulated over periods of minutes to hours, days to weeks, and months to years. Thus, signal transduction pathways such the Keap1-Nrf2 system can respond within minutes to transiently up-regulate antioxidant defenses and damage repair systems. Conversely, repeated or chronic exposure to stresses may blunt long-term defense and repair capabilities [43]. Then again, other interventions, such as exercise training or dietary restriction [132, 134, 237] have been shown to exert longer-lasting (mostly positive) effects that are, nevertheless, still transitory because they depend on maintenance of training or dietary protocols.

Our new understanding of Adaptive Homeostasis [132, 134] now provides a conceptual and mechanistic framework within which to more fully appreciate the implications of the Free Radical Theory of Ageing. In young and middle-aged organisms, Adaptive Homeostasis is proposed as a mechanism by which the effects of variations in numerous environmental and metabolic stresses, including oxidant exposure, are minimized by expanding the range of protective capacities at need. In contrast, aged animals and humans, during the final third of lifespan, experience diminished or dysfunctional adaptive homeostatic capabilities and accumulate stress-induced structural and functional damage at exponential rates. Increased levels of Bach1 and c-Myc may be at least partly responsible for loss of adaptive efficacy in advanced age, due to inhibition of the Nrf2 signaling pathway. Whether or not declines in the effectiveness of other protective signaling pathways also contribute to the ageing phenotype remains to be seen. Viewed then as a facet of Adaptive Homeostasis, along with many other age-related abatements in stress-adaptability, the Free Radical Theory of Ageing once again appears both viable and robust.

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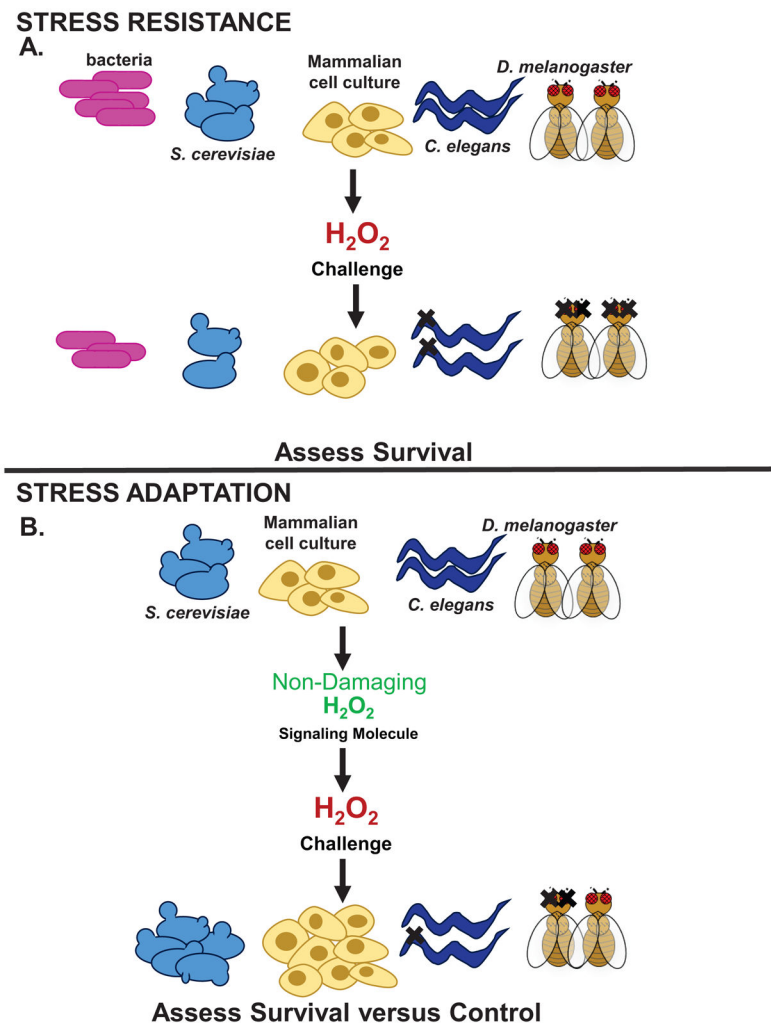


Figure 1. Assessment of the Stress Response

In studies assessing the ability of an organism to withstand an oxidative stress it is important to perturb the homeostatic set-point. (A) Stress resistance is commonly measured to assess tolerance. Biological models (yeast, mammalian cell culture, nematode worms, and fruit flies) are treated with a semi-lethal, high concentration of an oxidant to assess survivability or other markers of stress tolerance. One of the limitations of this approach is the overwhelming biological assault to the system, leading to extensive oxidative damage. Assessment of the adaptive stress response, enables an organism to first activate multiple protective pathways prior to being challenged with a semi-toxic concentration. (B) The adaptive response is induced in multiple biological systems (yeast, mammalian cell culture, nematode worms, and fruit flies) and is carried out by treating the organism with a non-damaging, signaling concentration of an oxidant to stimulate stress-inducible pathways (including those transcriptionally regulated by Nrf2). Once pathways are activated and upregulated, the organism is then challenged with a semi-lethal dose of an oxidant, and subsequently survival and other markers of stress activation are measured.

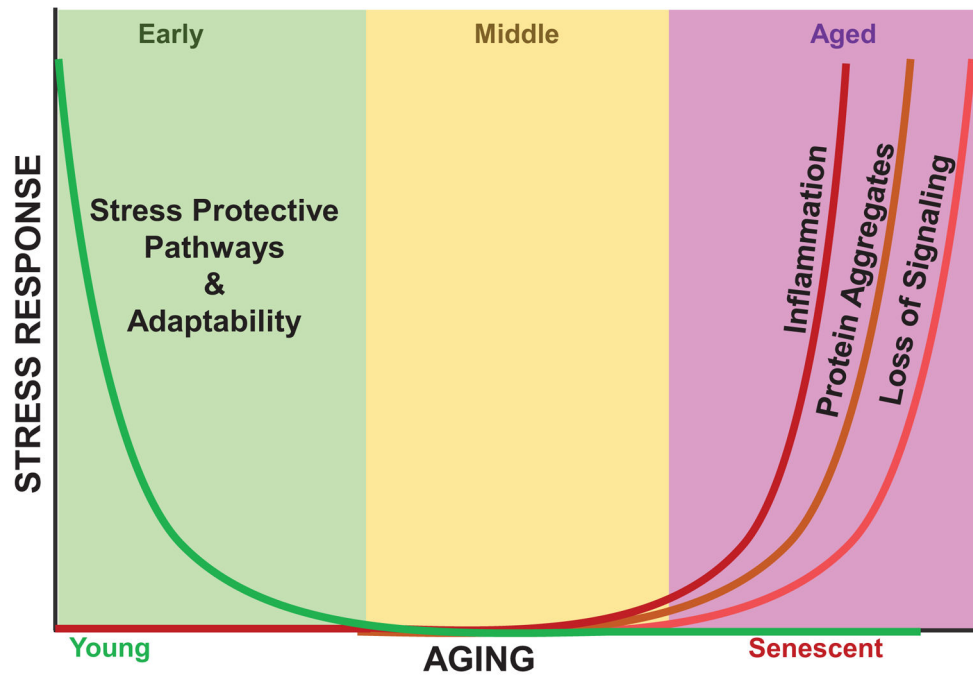


Figure 2. Age-Dependent Decline of the Adaptive Stress Response

Early passage cells and young organisms are capable of rapidly and transiently upregulating the adaptive homeostatic response. In turn, keeping damage accumulation, such as protein aggregates, limited and cellular maintenance. However, with age, the inducibility of the adaptive response declines, which is accompanied with a parallel rise in protein aggregation, sluggish cellular signaling and basal rise in inflammation ('inflammaging').

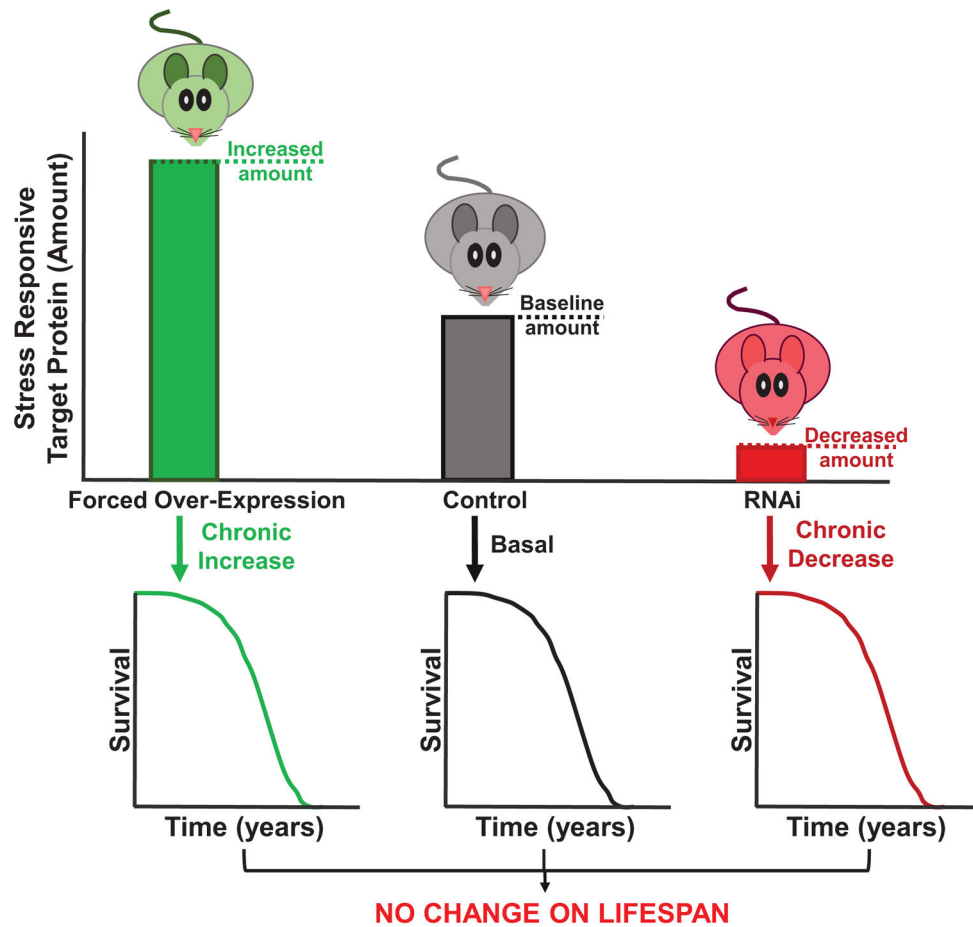


Figure 3. Chronic Up- or Downregulation of Target Protein Does Not Extend Lifespan

Chronic up- or downregulation of multiple stress-responsive proteins have shown little consistent impact upon lifespan across multiple biological models. Forced overexpression (OE) or suppression (RNAi), chronically throughout the lifespan, may limit the ability of transient modulation of target protein to appropriately respond to a stimulus. Instead, by continually upregulating or downregulating a target protein, creates a new physiological set-point, and may limit the dynamic stress-responsive capability within the cellular environment. Thus, many efforts for chronic changes in stress-responsive proteins have shown no impact upon lifespan.