Published in final edited form as:
J Clin Exp Pathol. ; Suppl 4: . doi:10.4172/2161-0681.S4-005.

Mouse Models of Oxidative Stress Indicate a Role for Modulating Healthy Aging

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Abstract

Aging is a complex process that affects every major system at the molecular, cellular and organ levels. Although the exact cause of aging is unknown, there is significant evidence that oxidative stress plays a major role in the aging process. The basis of the oxidative stress hypothesis is that aging occurs as a result of an imbalance between oxidants and antioxidants, which leads to the accrual of damaged proteins, lipids and DNA macromolecules with age. Age-dependent increases in protein oxidation and aggregates, lipofuscin, and DNA mutations contribute to age-related pathologies. Many transgenic/knockout mouse models over expressing or deficient in key antioxidant enzymes have been generated to examine the effect of oxidative stress on aging and age-related diseases. Based on currently reported lifespan studies using mice with altered antioxidant defense, there is little evidence that oxidative stress plays a role in determining lifespan. However, mice deficient in antioxidant enzymes are often more susceptible to age-related disease while mice overexpressing antioxidant enzymes often have an increase in the amount of time spent without disease, i.e., healthspan. Thus, by understanding the mechanisms that affect healthy aging, we may discover potential therapeutic targets to extend human healthspan.

Keywords

Aging; Oxidative stress; Healthspan

Introduction

Aging is characterized by the progressive loss in systemic and physiological functions that increase the likelihood for disease and death [1]. Observed changes in physiologic function include loss of pigments in the hair, wrinkling of the skin, bone fragility [2], muscle atrophy [3], brain atrophy [4], cancer [5-7] and organ dysfunction [8]. An increase in oxidative stress

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with age has been implicated to be at the root of many of these age-related physiologic declines and is the basis for the free radical theory of aging as presented by Denham Harman in 1956 [9]. This theory postulates that reactive free radicals produced by physiological processes induce aging through damage to macromolecules [9]. The theory was later modified to reflect the fact that hydrogen peroxide, peroxynitrite, lipid peroxides and reactive aldehydes are not radicals, but are strong oxidants and hence generated the oxidative stress theory of aging [10,11]. Oxidative stress-dependent aging is proposed to occur through an imbalance in the oxidant to antioxidant ratio, which leads to the damage in lipids, DNA and proteins that occurs with aging [12,13]. Although there is evidence in support of the oxidative stress theory of aging, especially in invertebrates, there are also vertebrate studies that do not directly support this theory [14-18]. This review will focus on the information gained from studies of mice regarding the role of antioxidant systems in aging.

Mitochondrial Function, Oxidative Stress and Oxidative Damage

Oxidative stress is defined as an imbalance between oxidants and antioxidants [12,13]. Elevated oxidants result in damage to macromolecules including 1) cytotoxic proteins that aggregate or perform toxic functions, 2) lipid oxidation that produces cytotoxic intermediates and final oxidation products that can damage proteins and DNA and induce cell death, and 3) modifications of DNA (mitochondrial and nuclear) leading to mutagenesis that produce gain/loss of function. Because mitochondria are the master control center for ATP production and thus a major source of ROS production, Denham Harmon in 1972 pointed to the role of mitochondria in aging and described the mitochondrial theory of aging [19].

Mitochondria account for the majority of oxidant and free radical production [19] through oxidative phosphorylation and formation of an electrochemical gradient that regulates both the formation of ATP from ADP and NAPDH from NADH. The flow of electrons across the electron transport chain from the oxidation of both NADH and FADH2 induces proton pumping from the mitochondrial matrix to the intermembrane space. The resultant increase in protons pumped to the intermembrane space forms the electrochemical gradient termed ‘proton motive force’ that regulates the production of ATP by ATP synthase [20], NADPH by NNT [21], and heat generation by uncoupling proteins [22]. NNT utilizes the energy of protein motive force in the mitochondria to convert NADH to NADPH which requires energy of activation as is observed with ATP synthase converting ADP to ATP. Electrons can escape the respiratory chain to form superoxide, which can be readily converted to hydrogen peroxide in the presence of MnSOD in the mitochondrial matrix [23] and CuZnSOD in the intermembrane space [24]. The generated hydrogen peroxide can diffuse out of mitochondria [25] and oxidize proteins [26,27] lipids [28], and DNA (nuclear [29], mitochondrial [30]) or can be degraded to oxygen and water by peroxisomal catalase, the glutaredoxins, peroxiredoxins, thioredoxins, and glutathione peroxidases [18]. Unscavenged superoxide can react with nitric oxide to produce peroxynitrite [31], which increases nitrative stress [32].

Peroxynitrite, like hydrogen peroxide, can modify proteins [33-40] and induce formation of lipid modifications [33,34,41-43]. Peroxynitrite can also cause modifications in both nuclear and mitochondrial DNA [35-37]. Other mechanisms of damage to macromolecules can occur as a result of free iron [44,45] and copper [46-47] through Fenton chemistry [48]. Likewise, hydrogen peroxide and superoxide reacting through the Haber-Weiss reaction [39] can cause oxidative damage. Thus, there are numerous sources of oxidant stress and the potential for oxidative damage is significant.

While oxidant stress in the mitochondria may be hypothesized to have negative consequences, in the cytosol hydrogen peroxide is suggested to induce necessary redox-dependent signaling molecules [25] designed to either bolster antioxidative defenses (Keap1-NRF2 axis) [49], induce apoptosis and energy control (GSK3β [50-51], JNK [52-55], ERK-1,2 [55], p38 [55], p53 [56]), increase proliferation [57], differentiation [57], apoptosis [50-54,56] or necrosis as ascribed to glutathione redox couples [58-60]. Oxidation of the cysteine residues of many signaling molecules is important for cell signaling processes, and it has become apparent that reduced oxidative stress can impair signaling cascades for proliferation and differentiation [57]. On the other hand, too much oxidative damage induces aberrant apoptosis or even necrosis, suggesting a need for the right amount of oxidants for proper survival and growth as well as death. Thus, reactive oxygen species can have divergent effects, causing damage to the cell while inducing signaling that may be important for maintaining cellular function. In summary, even though oxidant damage accumulates with age and can have detrimental effects, some amount of oxidative stress signaling is beneficial for physiological function.

Modulation of Antioxidant Status Impacts Life and Healthspan

Several groups have attempted to address the oxidative theory of aging by asking whether transgenic and knockout models of enzymes involved in detoxification of oxidants alter lifespan. There are three main groups of cellular antioxidants: 1) autocatalytic antioxidants that do not require reducing substrate but rather cycle between oxidation and reduction intermediates (catalase and superoxide dismutases), 2) antioxidants that couple to glutathione through glutathione reductase with NADPH (glutaredoxins and glutathione peroxidases), and 3) antioxidants that couple thioredoxin reduction through thioredoxin reductase with NADPH (methionine sulfoxide reductases, peroxiredoxins and thioredoxins). Proper function of the antioxidant system as a whole is more complicated than just the expression of one of the many enzymes. For example, antioxidant redundancy may compensate for the loss in one of the enzymes. Despite this complexity, inhibition of oxidant stress may be beneficial to vertebrates, protecting them from pathologies and age-related disability and increasing healthy living, or healthspan. Healthspan is generally defined as the portion of life spent without overt disease and disability [61]. The following sections attempt to address how antioxidant knockout and transgenic models might affect both life- and healthspan in mice.

Examples of mouse models lacking antioxidant enzymes can be identified that are embryonic lethal [14,62-68], shorten lifespan [14,69,70], or have no effect (Table 1). Several antioxidant deficient models have been reported to have deleterious effects on

healthspan [14,68,71-99]. Remarkably, in one case, a reduction in antioxidant defense (Gpx4+/- mice) actually increases lifespan significantly by 7% [14,100]. A handful of models appear normal [68, 79,82,86,105], suggesting the important role for redundancy in components of antioxidant defense in mice and vertebrates [77,106]. Most mouse models with increased antioxidant defense show no effect on lifespan, although several studies report improved healthspan [14,63,87,107-132], and a few report increased lifespan [111-113] (Table 2). Many of the transgenic mice overexpressing proteins important in antioxidant defense demonstrate marked resistance to acute stressors, supporting a role for antioxidant defense in healthspan [14,87,112,122,130,133-134]. In the following sections we discuss the importance of these results with respect to aging and disease.

Antioxidant knockout mice with lethal phenotypes

Lethal phenotypes (Table 1) provide important information about the role of either the antioxidant or oxidant species in the development of the organism; however, they do not allow investigation of age-related effects. Thus, going forward, models of conditional deletion of these critical enzymes would be valuable in determining whether these deletions reduce health in the adult animal. Understanding the roles of the critical antioxidant enzymes through the use of conditional knockouts will provide insight into the function of these enzymes in physiological aging.

MnSOD

MnSOD catalyzes the autocatalytic oxidation and reduction of superoxide to both molecular oxygen and hydrogen peroxide. MnSOD is the primary line of defense in the mitochondria as a scavenger of superoxide produced by the electron transport chain. MnSOD deficiency therefore would be predicted to result in mitochondrial damage and loss of mitochondrial function as superoxide is damaging itself and also produces the strong oxidant hydroperoxyl radical. Complete loss of the gene encoding MnSOD results in a lethal phenotype with some live births, but these are short-lived depending on the mouse strain [62,135-136]. In contrast, mice heterozygous for MnSOD have increased oxidative damage [72,90-92,137-139] but normal lifespan [14] and increased cancer incidence [72]. MnSOD heterozygous knockout mice also have increased mitochondrial dysfunction in heart [90]. Thus, complete loss of MnSOD is lethal, but a 50% reduction in MnSOD has only moderate effects, suggesting a critical threshold of oxidation in the mitochondria by superoxide.

The importance of MnSOD is further underscored by studies using the conditional deletion of MnSOD in specific tissues like liver [140] and muscle [140]. The conditional knockout of MnSOD targeted to muscle led to increased oxidative damage and reduced aerobic capacity [141]. Recently, a study found that a conditional MnSOD knockout in motor neurons resulted in remarkably low levels of oxidative damage but increased the disorganization of motor neurons [142]. Another paper suggests that MnSOD upregulation in tumors leads to increased metastasis and invasion, which supports the above model [143]. Taken together, the data suggest that MnSOD is critically important for proper mitochondrial function and that future studies using conditional knockouts of MnSOD, particularly in neurons, might provide a basic understanding of mitochondrial superoxide tolerance.

Phospholipid hydroperoxide glutathione peroxidase (Gpx4)

Gpx4 catalyzes the reduction of lipid hydroperoxides at the expense of the reductant GSH. Gpx4 is the most abundant lipid hydroperoxide reductase and is shared among the mitochondria, nucleus and cytosol [144]. Deletion of Gpx4 is embryonic lethal [63] and recently, it was shown that cre-recombinase-dependent ablation of Gpx4 results in the death of adult mice in two weeks, underscoring the critical importance of this enzyme [146]. Gpx4 reduces lipid hydroperoxides and hence terminates species that can mitigate lipid peroxidation propagation reactions. The ability to reduce lipid hydroperoxides for cellular membrane signaling and for mitochondrial cardiolipin [110] activity is required for proper cellular function as evidenced by increased apoptosis in $Gpx4^{+/-}$ mice with reduced cancer incidence [100]. Gpx4 heterozygous mice have increased oxidative damage with age [14,100,110,145]. Although Gpx4 deletion is embryonic lethal, the heterozygous mice have a mild increase in median lifespan [100]. Quite strikingly, these mice also had no incidence of impaired glomerulonephritis and a reduction in age-dependent lymphomas [100]. This unexpected increase in lifespan in response to reduced Gpx4 activity may be due to sensitization to cell death, suggesting a selection for cells and mitochondria with better physiologic function, which is supported by a reduced cancer incidence [14]. It is possible that the reduction in Gpx4 activity in these mice leads to a compromise in lipid integrity of cellular membranes and mitochondria. Recent reports suggest that Gpx4 conditional ablation in the brain leads to increased mitochondrial damage to lipids as measured by increased levels of 4-hydroxynonenal [146], an end product of lipid peroxidation termination reactions. This is important as lipid hydroperoxides compromise the mitochondrial lipid membrane and potentially cardiolipin, leading to initiation of the mitochondrial permeability transition and activation of caspase-dependent cell death through Apaf1 [147]. It is possible that mitochondria producing excessive oxidative stress undergo lipid peroxide-dependent increases in the mitochondrial permeability transition and apoptosis. Hence, a higher sensitivity of mitochondria to lipid peroxidation propagation reactions might select for removal of poor performing mitochondria and cells, thus selecting for cells that have better functioning mitochondria. Consistent with this, Gpx4+/- mice have elevated levels of cytosolic cytochrome c indicating elevated activity of the mitochondrial permeability transition [146]. Also, a reduction in tumor incidence in these animals might be supported by increased sensitivity to apoptosis. Although the role of lipid hydroperoxide in apoptosis is still not completely understood, the Gpx4+/- and Gpx4 conditional knockout mice provide evidence supporting a mechanism involving mitochondrial membrane disruption and permeable transition for apoptosis. Additional studies are required to understand the role of Gpx4 in embryonic development and in the adult mouse.

Thioredoxin and thioredoxin reductase

Thioredoxins catalyze the reduction of oxidized antioxidant enzymes MSRA/B and peroxiredoxins as well as the reduction of oxygen-centered oxidants and radicals to oxidized thioredoxin. Oxidized thioredoxin cysteine residues are then reduced back by reducing substrate NADPH in the presence of thioredoxin reductase. Thioredoxin 1 (Trx1) and thioredoxin reductase 1 (TR1) regulate cysteine reduction in the cytosol while thioredoxin 2 (Trx2) and thioredoxin reductase 2 (TR2) regulate cysteine reduction in the mitochondria. Both Trx1 [65] and TR1 [66] knockout mice are embryonic lethal. Recent evidence suggests

that Trx1 controls ASK1-dependent apoptosis in cells [148-149], suggesting it is necessary for cellular selection in embryonic development. Reduction of oxidized thioredoxin in the cytosol is further required for maintenance of ASK1-dependent apoptosis [148]. Therefore, it may be that the lethal phenotypes of Trx1 and TR1 are related to the inability to control cellular death pathways that are constitutively active. Likewise, mice null for either Trx2 [64] or TR2 [67] are embryonic lethal, suggesting that these enzymes are required for embryonic development. Thioredoxin 2 is required for maintaining methionine sulfoxide reductase-A and -B (MSRA, MSRB) [150-152] and the peroxiredoxin [153] superfamily redox state in the mitochondria. A report showed that $Trx2^{+/-}$ mice have an insignificant, 16% reduction in maximal lifespan and insignificant, 7% reduction in median lifespan although it should be noted and is unfortunate that the cohort contained a small number of animals [14]. These $Trx2^{+/-}$animals also had increased damage to macromolecules and reduced mitochondrial function [14]. Thus, the relative importance of these enzymes in the mitochondria as it relates to aging is not known.

Antioxidant knockout/heterozygous mice that reduce lifespan

Mouse models with reduced antioxidant enzyme activity that result in a reduced lifespan (Table 1) provide indirect support of the oxidative theory of aging. There are four gene deletions that have been reported to impact lifespan negatively (i.e., Prx1 and -2 [14,68-69], CuZnSOD and MsrA).

Peroxiredoxin (Prx)

conventional Prxs (Prx1, 2) [158] catalyze the reduction of oxidants through the random oxidation of their two peroxidatic cysteine residues and resolving with the non oxidized cysteine residue (Prx-2S-H → Prx-S-OH + -SH → Prx-S_2). Evidence also indicates that Prx1 interacts and stabilizes in the inactive state p66Shc protein [155] with similar mechanisms to that observed between Trx1 and ASK1. However, there are also two other distinct peroxiredoxins which include the one cysteine peroxiredoxins and the non conventional two cysteine peroxiredoxins which are reviewed in [159]. The one cysteine peroxiredoxin (Prx6) catalyzes the reduction of oxidants by utilizing the reductive power of vitamin C or by coupling to the thioredoxin redox couple by reducing the sulfenic acid intermediate back to the sulfhydryl [156]. Under extreme oxidant stress, the sulfenic acid residue can oxidize further to the sulfinic (R-SO_2-H) and sulfonic acid (R-SO_3-H) and hence suggesting its sensitivity to bolus oxidant stress [157]. The exact mechanism is suggested to flow from the oxidation of its cysteine residue to sulfenic acid and consequent condensation of the sulfenic acid with the reduced antioxidant sulfhydryl residue forming a disulfide and water (Prx-SH → Prx-S-OH + Trx-SH → H2O + Prx-S-S-Trx-SH → Prx-SH + TrxS_2) [158]. As for the non conventional two cysteine peroxiredoxins (Prx5) [158], these enzymes do not use the classical two cysteine peroxidative activity but rather use a single cysteine residue with peroxidative activity and utilize the other cysteine as a resolving cysteine residue (R-S-OH (peroxidative) + R-S-H (resolving)→ R-S-S-R) while the typical conventional utilizes the same mechanism only both cysteine residues have peroxidative activity suggesting a fifty percent chance of being either the peroxidative cysteine or the resolving cysteine residue [158]. Mice deficient in Prx1 are shown to have a 15% reduction

in lifespan [69]. Pathologies observed in this knockout model included hemolytic anemia [69], cardiovascular atherosclerosis [98], allergic asthma traits/abnormal lung inflammation to acute challenge [99] and cancer [69]. Localization of Prx1 appears to be mostly nuclear and results in a reduction in antioxidant capacity in the nuclear compartment [159]. Although a 15% reduction in lifespan was reported in these Prx1 knockout mice, the question becomes whether these animals died because of lack of antioxidant defense or because of reduced antioxidant-induced aggressive tumor formation.Likewise, reduced mitochondrial Prx2 also reduced lifespan by 15% [70, 160]. The reduction in lifespan observed in both of these models suggests the importance of peroxiredoxins in the regulation of redox homeostasis. Pathologies observed in the Prx2 knockout mice include cardiovascular disease [96], hemolytic anemia [70], inflammation [161], senescence [162], inhibited immune function [163], and enlarged thymus [164], suggesting an elevated inflammatory response with age.

CuZnSOD

Like MnSOD, CuZnSOD catalyzes the autocatalytic oxidation and reduction of superoxide to molecular oxygen and hydrogen peroxide in the cytosol. As seen in the Prx1 and Prx2 knockout mice that have increased cancer with age, CuZnSOD knockouts have increased hepatocellular carcinoma with age [73]. The reduction in lifespan (30%) is much more pronounced in CuZnSOD knockout mice than in Prx1 and Prx2 knockout mice. CuZnSOD knockout mice also have severe pathologies, including muscle atrophy [101,103-104, 165], bone fragility [166], axonal degeneration [167], loss of hearing [168], β-islets dysfunction [169], reduced wound healing [170], cataracts [171], macular degeneration [172], vascular hypertrophy [173,174], and infertility. Taken together, the data from antioxidant knockouts of Prx1, Prx2 and CuZnSOD might provide some support for the oxidative theory of aging.

Methionine sulfoxide reductase A

(MsrA). Mammalian species have developed a systematic mechanism to degrading easily oxidized methionine residues. There are two enzyme subclasses which degrade the R- or S-enantiomer (epimer). MSRA is responsible for the degradation of S-methionine sulfoxide whereas MSRB is required for the reduction of R-methionine sulfoxide [71]. Both enzymes catalyze the reduction of methionine sulfoxide reductase by coupling a three cysteine electrophilic backside attack on the sulfoxide residue forming a mixed disulfide and finally reduction by the second cysteine sulfur residues to release water and reduced methionine sulfoxide [154]. The final reduction of methionine sulfoxide reductase is performed by coupling to thioredoxin and thioredoxin reductase [154] at the expense of NADPH reducing substrate. In addition, one research group showed a reduction in lifespan of 40% in the methionine sulfoxide reductase A (MsrA) knockout mice [89]; however, the data are difficult to interpret as the lifespan of the control group was also reduced as compared to known lifespan data for C57Bl6 mice [14]. Furthermore, our group has recently shown that the MSRA KO animals do not have reduced lifespan [14,71]. Likewise, mice deficient in MsrB, which reduces the R enantiomer of methionine sulfoxide, also show no difference in lifespan compared to wild type mice up to 20 months of age [74].

Antioxidant knockouts that have no effect on lifespan but are detrimental to healthspan

Although knockout models of antioxidant enzymes in mice have demonstrated that specific types of oxidative stress might be related to aging, most studies show pronounced effects on healthspan, which suggest their importance for healthy aging (Table 1). Evidence in support of this is formation of pathologies includes cancer, cataracts and lung dysfunction. Hence, oxidative stress-induced signaling may be important in contributing to maximal cellular function and may partially explain some of the ambiguous beneficial effects on aging in overexpressing models.

Glutathione peroxidase

(Gpx). Glutathione Peroxidases catalyze the reduction of oxidants like hydrogen peroxide, peroxynitrite and lipid hydroperoxides to the oxidation of glutathione. The products include the formation of water/nitrite/alcohols and oxidized glutathione. Oxidized glutathione is reduced in the presence of glutathione reductase to glutathione (GSH) at the expense of reducing equivalent NADPH. Quite strikingly, Gpx1 knockout have no apparent effect on lifespan [14]. Gpx1 knockout mice were shown to have reduced ovalbumin-induced allergic asthma [175], increased cataracts [176], and hypertension [177], sensitivity to ischemia reperfusion injury and infarct size [178-179], and sensitivity to diquat and paraquat [180-181]. Gpx1 knockout in human APP transgenic mice have accelerated Alzheimer's pathology [182]. Gpx2 and -5 lifespans were not reported; however, Gpx2 null mice have numerous pathologies including intestinal cancer [77,183] and increased lung inflammation after challenge [184], while Gpx5 have reduced fertility in males [76]. Although there appears to be reproductive problems in the Gpx5 knockout mice, the relative necessity for these specific three enzymes appears to be redundant and may not be required for maximal protection against oxidants during aging.

Glutaredoxin (Grx)

Grxs are enzymes that utilize active cysteine and selenocysteine residues to form mixed disulfides in oxidized proteins or are oxidized directly with oxidants and then utilize glutathione redox chemistry to form oxidized glutathione and finally reduced Grxs. In conjunction with the glutathione peroxidase enzymes, the Grx1 and Grx2 enzymes have no apparent or reported effect on lifespan. Grx1 knockout mice show reduced lung function [86], sensitivity to paraquat and diquat [82], increased cataracts and lens dysfunction [83], as well as reduced angiotensin-2-dependent cardiac hypertrophy [79]. Grx2 knockout mice, like Grx1, have impaired lens function [88]. The reduced lung function and increased cataracts in the Grx1 and Grx2 mice might suggest sensitivity to elevated oxygen levels as both organs are in proximity with atmospheric oxygen.

Peroxiredoxin (Prx)

As mentioned earlier, Prx catalyzes the reduction of oxidants and protein disulfides through multiple mechanisms that include thioredoxins for the two cysteine/selenocysteine Prx and vitamin C for the single cysteine Prx. Recently, Prx3 knockout mice were shown to have

increased susceptibility to LPS-induced inflammation [185]. Prx4 knockout mice also were reported to have elevated oxidative stress and cell death in spermatogenic cells, suggesting its importance in reproduction [186]. Prx6 knockout mice are susceptible to oxidative damage [116]. Furthermore, many clinical manifestations were observed in these mice that include susceptibility to oxidant stress by cigarette smoke [85], paraquat [115], or hydrogen peroxide [187], as well as increased atherosclerosis [188-189] and reduced wound healing [127]. Prx6 is a unique enzyme as it has both phospholipase activity and peroxidase activity [190], making it an intriguing enzyme for both lipid-mediated inflammation and antioxidant defense. The data suggest that Prx3, -4 and -6 are important for protection against oxidative challenge and that Prx6 may be important for phospholipid signaling. Lifespan studies on these knockout animals may provide insight into the importance of the peroxiredoxin superfamily of antioxidant enzymes during aging. At the same time, these enzymes may be redundant in nature as compared to the more abundant Prx1 and Prx2.

Catalase

Catalase catalyzes the autocatalytic oxidation and reduction of hydrogen peroxide to molecular oxygen and water. Studies using catalase knockout mice have shown that peroxisomal overexpression of catalase has no effective outcomes on lifespan or healthspan [14]. With age, catalase expression and activity are reduced in mouse brain [191]. One can surmise that the compartment where the catalase normally resides provides no mechanistic insights into protective functions against aging, although the peroxisome is important for regulation of the pentose phosphate pathway (NADPH substrates), catabolism of fatty acids, and is suggested to be involved in cholesterol and isoprenoid biosynthesis [192].

Extracellular SOD (EcSOD)

Like MnSOD and CuZnSOD, EcSOD catalyzes the autocatalytic reduction and oxidation of superoxide to hydrogen peroxide and oxygen in the extracellular compartment. Lack of EcSOD has no deleterious effects on lifespan like those found in mice null for MnSOD or CuZnSOD. Deficiency of EcSOD results in sensitivity to acute stressors like hypoxia induced right and left ventricular hypertension and hypertrophy [75,193-194]. Likewise, EcSOD knockout mice have renal reperfusion injury [195], emphysema [87], lung sensitivity [196,197], and abnormal corneal function [198].

Overexpression of antioxidants that increase lifespan/median lifespan

Overexpression of antioxidants in transgenic mice is the most powerful method to test the oxidative stress theory of aging. One might predict that increased protection from oxidants would extend the amount of time required to accumulate the same levels of macromolecule damage as that obtained under normal conditions, and hence benefit lifespan. The healthspan effects of the antioxidant-overexpressing enzymes can be significant and will be discussed in the next section (Table 2). In addition, it should be noted that very few lifespan data on antioxidant transgenic animals have been reported and need to be determined.

Thioredoxin (Trx)

As stated earlier, thioredoxins center on the reduction of oxidants, oxidized proteins and oxidized antioxidants, making them important for redox control. Transgenic mice overexpressing Trx1 originally were suggested to have extended lifespans [199]. Contrary to these findings, a recent article from our laboratory assessed the life extending properties of Trx1-overexpressing mice and found that Trx1 overexpression did not extend maximum lifespan but improved median lifespan, which may be surmised to be indicative of increased healthspan [200]. Trx1-overexpressing mice were also shown to be resistant to multiple acute stressors. They also are protected against chronic myocardial infarction [201], diabetic embryopathy [202], streptozotocin-induced diabetic osteopenia [203], chronic pancreatitis [204], apoptosis in alveolar walls, lung dysfunction [205] and cigarette smoke-induced inflammation and emphysema [206]. On the other hand, Trx1 transgenic mice have elevated skin cancer incidence [196], suggesting a negative consequence of a reduction in apoptosis that is controlled by apoptosis signaling cascade 1 (ASK1)-Trx1 redox axis [148]. The overall benefits of thioredoxin overexpression on healthspan appear to be beneficial against stressors, although the increased incidence of skin tumors should be addressed.

Mitochondrial catalase (MCat)

The overexpression of MCat has been shown to increase lifespan of mice [207]. Although catalase is not normally expressed in mitochondria, mitochondrial expression of catalase presumably reduces hydrogen peroxide stress and potentially reduces the reliance on antioxidant coupled NADPH-driven reduction of oxidants in the mitochondria, thereby maintaining a higher availability of NADPH for exogenous antioxidant reduction. The increase in lifespan in MCat mice is also coupled to increased healthspan as evidenced by a reduction in the following acute pathologies, including cardiac myopathy in mutator mice [208-209] and antiretroviral-dependent cardio myopathy [210-211], invasive breast cancers [212], and aging-dependent pathologies [213].

In summary, data obtained from a few antioxidant enzyme knockouts and transgenic mice studies appear to provide substantial evidence towards certain key metabolic pathways that are important for lifespan in mice. One pathway that seems to play a major role in lifespan as observed in the Gpx4 heterozygous mice and the Trx1 transgenic mice is the sensitivity of cells to apoptosis. It appears that the sensitivity of cells to apoptosis selects healthy cells (Gpx4+/- mice) and that a reduction of this pathway leads to transformation and cancer, reducing the ability of these mice to achieve increases in optimal health. This partially explains the increase in median lifespan in Trx1 transgenic and Gpx4 heterozygous mice. It also is evident that the introduction of an autocatalytic enzyme into the mitochondria that is predicted to spare mitochondrial substrates increases lifespan in mice, suggesting that mitochondria redox control may be important for life-and healthspan.

Antioxidant transgenic mice that do not affect lifespan but improve healthspan

Although reduced expression of most antioxidant enzymes do not systematically decrease lifespan, can increased expression of antioxidants induce healthy aging? From the literature,

a number of studies in transgenic mice overexpressing antioxidant enzymes show improved healthspan (Table 2). The majority of studies analyzed report changes in cardiovascular function, lung function, neurodegeneration, cancer, and diabetes.

Peroxisomal and nuclear catalase

Peroxisomal catalase transgenic mice have been reported to have a 4% non significant increase in lifespan, and nuclear-targeted catalase had no effect on lifespan [214]. Catalase overexpression in the peroxisomes can protect against hypertension and tubular apoptosis in angiotensinogen transgenic mice [215] and lead to improved cardiac contractility in type 1 and type 2 diabetic mice [216]. Nuclear targeted catalase sensitized carcinoma cells to bleomycin and increased damage to DNA with paraquat but protected against DNA damage from hydrogen peroxide. DNA damage was unaltered in normal aging mice [217-218], but based on results from paraquat treatment, the data suggest sensitivity to nuclear superoxide.

Glutathione peroxidase (Gpx)

The effect of increased Gpx1 expression on lifespan has not been reported. However, Gpx1 transgenic mice have been shown to have some pathology, including an increased diabetic phenotype as measured by insulin resistance and obesity [123]. These mice are protected against angiotensin-II-dependent vascular dysfunction [219], inhibition of cerebroarteriole vasodilation to arachidonic acid [220], protection from left ventricular remodeling after myocardial infarction [221], and neuroprotection against ischemia reperfusion injury [222]. A plausible reason for Gpx1 inducing a diabetic phenotype may be centered on disruption of glutathione redox and the limiting NADPH substrate-dependent reduction of GSSG by glutathione reductase. The phospholipid hydroperoxide glutathione peroxidase (Gpx4) transgenic mice show improvement in multiple parameters associated with good health including improved cardiovascular function and reduced atherogenesis [223], age-dependent losses in mitochondrial function [107,110], amyloid toxicity and Alzheimer's pathology [108, 111], and retinal lens protection from oxidative damage [224]. These data indicate that both enzymes protect against acute challenges and improve overall healthspan outcomes in mice.

Glutaredoxin (Grx)

As was observed in the Gpx1 and -4 overexpressing mice, Grx1 and -2 overexpression protects against infarct size after injury [80,120]. Grx2 overexpressing mice are also protected from doxorubicin-dependent cardiac injury [225], while Grx2 protects against motor neuron apoptosis and progression of Amyotrophic Lateral Sclerosis (ALS) [226]. The following data suggest that both Grx1 and Grx2 protect against acute challenges. These data support increased antioxidant defense as a benefit to healthy aging. The lifespans for neither Grx1 nor -2 overexpressing mice have been reported.

Peroxiredoxin (Prx)

Lifespans of Prx-3, -4 and -6 transgenic mice are not currently reported. Prx3 transgenic mice are known to have increased healthspan outcomes and include improved glucose tolerance in mice [132] and a reduction in left ventricular remodeling [125]. Prx4 transgenic

mice also show a reduction in diabetes after challenge with a toxin, suggesting insulin-secreting beta cells are protected against oxidative stress in these mice [129]. Transgenic mice for Prx6 have increased healthspan that includes improved cardiovascular function from ischemia reperfusion injury [189] and from atherosclerosis [188], as well as wound healing [132]. They also have reduced lung injury to hyperoxia [227] and hydrogen peroxide [187], protection against diet-induced hydrogen peroxide in the aortic root [119] as well as protection against alcohol-dependent hepatic lipid accumulation [118]. On the other hand, the Prx6 transgenic mice do not protect against diet-induced atherosclerosis, suggesting that chronic stress may not be protective in many of these overexpressing enzymes or that Prx6-dependent phospholipase activity has a negative impact on inflammation by increasing the secretion of prostaglandin synthase-and lipoxygenase-dependent substrates. The data obtained from the transgenic Prx superfamily of enzymes suggest protection against acute stressors but more research is needed for chronic stressors.

Methionine sulfoxide reductase A (MSRA)

The effect of increased MSRA expression on lifespan has not been reported in the MSRA transgenic mice. As seen in multiple antioxidant overexpressing mouse models, MSRA overexpressing mice had reduced ischemia reperfusion injury [112], and it was reported that MSRA requires myristoylation (membrane targeting) for its functional benefits. This may be through protection of membrane bound signaling molecules from methionine oxidation, which might reduce the signaling in the membrane or MSRA might require hydrophobic interaction for activity. Further research into the function of MSRA in healthspan and lifespan needs to be addressed.

Cytosolic (CuZnSOD), mitochondrial (MnSOD) and extracellular (EcSOD) SOD

Both MnSOD and CuZnSOD transgenic mice show no increase in lifespan [14]. Overexpression of MnSOD in mice appears to have no effect on physiologic functions mediated by irradiation damage in the lungs [228]. In skeletal muscle, MnSOD transgenic mice preserve mitochondrial function and myoblast function with age but fail to preserve muscle mass [229]. MnSOD transgenic mice also attenuate cardiac myopathy in diabetic mice [117]. Overexpression of CuZnSOD appears to have no effect on muscle function, or on exposure in whole animals to paraquat or gamma irradiation [122]. It was also observed that CuZnSOD, MnSOD, or catalase overexpressing mice had no effect on diaphragm contraction-dependent fatigue or recovery from fatigue [230]. Quite strikingly, CuZnSOD in combination with both EcSOD and/or Gpx1 overexpression is able to block xanthine/hypoxanthine oxidase-dependent damage to islets [231]. Likewise, triple transgenic mice for Gpx1, CuZnSOD, and EcSOD are able to improve blood glucose control in streptozotocin-induced diabetic animals as compared to EcSOD or Gpx1 transgenic mice [231]. Overexpression of EcSOD was able to reduce cardiovascular abnormalities after ischemia reperfusion injury [134] and to inhibit hyperoxia-induced damage to the lungs [232-234]. Taken together, these data indicate that the overexpression of CuZnSOD and MnSOD appears to be less beneficial than the EcSOD overexpression in terms of healthspan in mice. This may be partially explained by a reduction in extracellular superoxide under pro-inflammatory states that leads to oxidation of extracellular proteins and lipids and increased inflammatory signaling.

NADPH, The rate limiting redox substrate in the recycling of non autocatalytic antioxidant enzymes

As stated previously, the formation of radicals and oxidants that can damage cellular components. Mitochondrial superoxide and hydrogen peroxide are a primary source of oxidants and hence control of reactive oxygen species must be maintained by these redox couples in mitochondria, cytosol and extracellular compartments. As discussed earlier, many antioxidant systems including methionine sulfoxide reductases [38, 152], thioredoxin reductases [40,235-236], glutathione reductases [40,237], glutathione peroxidases [76,144,238-241] and peroxiredoxins [241-242] require NADPH substrate. Glutathione reductases and glutathione peroxidases utilize the reduction potential of glutathione to form oxidized glutathione while methionine sulfoxide reductases, and peroxiredoxins couple to thioredoxin oxidation. The final reduction of oxidized glutathione or thioredoxin is catalyzed by the oxidation of NADPH by glutathione reductase or thioredoxin reductase [236,243], respectively [244,245].

NADPH in the mitochondria is produced through the NNT by the proton motive force and from the isocitrate dehydrogenase, malic linked NADP enzyme [246] and glutamate dehydrogenase [247,248]. NADPH in the cytosol is primarily produced by the pentose phosphate shunt through two steps which includes glucose-6-phosphate dehydrogenase and 6-phosphoglucanate dehydrogenase, which accounts for 60% of the total NADPH [249]. This system is elevated under oxidative stress [247-248]. Upregulation of NADPH overproducing and overexpressed enzymes like glucose-6 phosphate dehydrogenase extends lifespan of *Drosophila melanogaster* by 40% [250]; however, this has not been shown in a mouse model. Utilization of transgenic mice that increase NADPH availability might shed light on the importance of cytosolic and mitochondrial NADPH on lifespan, healthspan and disease. Likewise, the overexpression of NNT, isocitrate dehydrogenase, malic linked NADP enzyme and glutamate dehydrogenase in the mitochondrial may provide information on mechanisms observed in the mitochondrial catalase overexpressing mice, considering this autocatalytic enzyme should spare NADPH substrates in the mitochondria. To date, these models have not been produced or utilized; however, it would be interesting to determine whether increasing NADPH overexpressing mechanisms in conjunction with antioxidant overexpressing transgenic mice would generate synergistic coupling of the reductive chemistry that might improve healthspan or even lead to increased lifespan. These experiments would be informative with respect to the importance of redox balance in lifespan and healthspan [251,252].

Perspectives and Future Directions: What Have We Learned from Antioxidant Knockout and Transgenic Mice?

The oxidative theory of aging has been under scrutiny for the past several years in part because many antioxidant knockout and overexpressing mouse models have had no effect on lifespan. Thus, it is increasingly apparent that the oxidative theory of aging may need some modification. Although mice deficient in CuZnSOD, Prx1 and -2 all have reduced lifespan, evidence suggests that this is more likely related to increased cancer incidence and not to an underlying alteration in the aging process. Likewise, current reports using

overexpression of antioxidant enzymes show only modest changes in median or maximal lifespan with exception of mitochondrial catalase overexpressing mice. On the other hand, increasing antioxidant defense appears to increase healthy aging phenotypes in mice. This is important to note because the increase in human lifespan with extended periods of time living with disability is a significant economic burden. Therefore, research focused on improving healthspan by reducing the percentage of life spent with disability is imperative. To address the goal of improving healthspan, one must understand the mechanisms by which antioxidant systems impinge upon healthy aging phenotypes as observed in the literature, which include: 1) natural selection of cells and mitochondria through apoptosis, 2) reduction of mitochondrial dependence on substrate limiting redox control (NADPH), 3) antioxidant redundancy, and 4) other functions of the antioxidant enzymes in question. To further support the above goal, antioxidant enzyme knockout mice and overexpressing transgenic mice that address key mechanisms of healthy aging need to be studied. Healthy aging studies should be carried out with stringent control of husbandry and facilities as to minimize artifacts of husbandry and facilities. Artifacts of husbandry and study conditions impair the scientific understanding of antioxidant models of healthy aging. Adhering to research guidelines will improve our understanding of antioxidants as they relate to our goal of increasing life spent in healthy aging.

Acknowledgments

This work was supported by the Institutional NIA Training Grant on the Biology of Aging for Research Scientists (RTH - NIA5 T32AG021890) and a National Institutes of Health – National Institute on Aging Grant (HVR – P01AG20591).

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Abbreviations

ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate

NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
FADH2	Flavin Adenine Dinucleotide
NNT	Nicotinamide Nucleotide Transhydrogenase
Gpx	Glutathione Peroxidase
Grx	Glutaredoxin
Prx	Peroxiredoxin
Trx	Thioredoxin
CuZnSOD	Copper, Zinc Superoxide Dismutase
MnSOD	Manganese Superoxide Dismutase
EcSOD	Extracellular Superoxide Dismutase
MSRA	Methionine sulfoxide reductase A
MSRB	Methionine Sulfoxide Reductase B
MCat	Mitochondrial Catalase
TR	Thioredoxin Reducta

Table 1

Antioxidant Knockout Mouse Life- and Healthspan.

Enzyme	Lifespan Effect	Healthspan	References
NNT$^{-/-}$	nd	Diabetic phenotype and impaired mitochondrial function	[251,252]
Gpx1$^{-/-}$	No change	Reduced allergic asthma, cataracts, hypertension, reperfusion injury-dependent infarct size, AD pathology.	[14,175-182]
Gpx2$^{-/-}$	nd	Increased intestinal cancers, and lung inflammation from acute challenges	[77,183,184]
Gpx4$^{-/-}$	Lethal	Reduced cancer risk. Increased apoptosis-natural selection.	[14,63,99,114,146]
Gpx4$^{+/-}$	↑7%	Reduced cancer risk. Increased apoptosis-natural selection.	[14,100,110,145]
Gpx5$^{-/-}$	nd	Male infertility.	[76]
Trx1$^{-/-}$	Lethal	Embryonic lethal.	[65]
Trx2$^{-/-}$	Lethal	Embryonic lethal.	[64]
Trx2$^{+/-}$	↓16% maximal ↓7% median	Increased oxidative damage to macromolecules. Reduced mitochondrial function.	[14]
TR1$^{-/-}$	Lethal	Embryonic lethal.	[66]
TR2$^{-/-}$	Lethal	Embryonic lethal.	[67]
Grx1$^{-/-}$	nd	Lung dysfunction, cataracts, sensitivity to redox cyclers, lens dysfunction, and reduced cardiac hypertrophy.	[79,82-83,88]
Grx2$^{-/-}$	nd	Lung and lens dysfunction.	[87]
Prx1$^{-/-}$	↓15%	Hemolytic anemia, atherosclerosis, allergic asthma and lung inflammation, and cancer.	[69,98,99]
Prx2$^{-/-}$	↓15%	Hemolytic anemia, cardiovascular disease, inflammation, senescence, inhibited immunity and enlarged thymus.	[70,96,160-164]
Prx3$^{-/-}$	nd	Increased LPS-induced inflammation.	[185]
Prx4$^{-/-}$	nd	Increased oxidative damage, spermatogenic cell death, and testicular atrophy.	[186]
Prx6$^{-/-}$	nd	Oxidative stimuli sensitive, increased atherosclerosis, and reduced wound healing.	[85,115-116,127,187-190]
MSRB$^{-/-}$	Normal	Increased oxidative damage.	[74]
PCat$^{-/-}$	Normal	Normal.	[14,191]
EcSOD$^{-/-}$	Nd	Hypoxia-induced hypertension/hypertrophy of the heart, renal injury, emphysema/lung sensitivity, and corneal dysfunction.	[75,87,193-198]
MnSOD$^{-/-}$	Lethal	Embryonic lethal.	[62,135-136]
MnSOD$^{+/-}$	Normal	Increased oxidative damage, cancer, and reduced cardiac and mitochondrial function.	[14,72,90-92,137-139]
MSRA$^{-/-}$	Normal	Increased oxidative damage.	[14,71,89,97]
CuZnSOD$^{-/-}$	↓30%	Hepatocarcinoma, muscle atrophy, bone fragility, axonal degeneration, loss of hearing, b-islet dysfunction (pancreas), reduced wound healing, cataracts, macular degeneration, vascular hypertrophy, and female infertility.	[73,101,103-104, 165-174]

Antioxidants with changes in life- or healthspan are presented. However, many antioxidant knockout models have not yet been generated, which includes glutathione peroxidase-3, -6, -7 and -8, glutaredoxin-3, -4, -5, -6, -7, and -8, glutathione reductase, peroxiredoxin 5, and thioredoxin reductase 3. nd not determined

Table 2

Antioxidant Transgenic Mouse Life- and Healthspan.

Enzyme	Lifespan Effect	Healthspan	References
$Gpx1^{+/0}$	nd	Diabetic with insulin resistance/obesity, Angiotensin-II vascular problems, cerebrovasodilation issues, neuroprotection.	[123,219-222]
$Gpx4^{+/0}$	nd	Reduced cardiovascular disease, amyloidosis, retinal lens and mitochondrial dysfunction.	[107-111,224]
$Trx1^{+/0}$	↑14% median	Increased skin cancer, protection from myocardial infarction, diabetic embryopathy, pancreatitis, and lung dysfunction	[196, 199-206]
$Grx1^{+/0}$	nd	Reduced infarct size.	[80,120]
$Grx2^{+/0}$	nd	Reduced infarct size, and doxorubicin-dependent cardiac injury as well as CuZnSOD1-dependent neurodegeneration.	[80,120,225-226]
$Prx3^{+/0}$	nd	Improved glucose tolerance, reduction in left ventricular remodeling and amyloidosis in APP transgenic mice.	[125,132]
$Prx4^{+/0}$	nd	Protection from toxin induced diabetes	[129]
$Prx6^{+/0}$*	nd	Reduced reperfusion injury, atherosclerosis, lung dysfunction and alcoholic fatty liver. Improved wound healing.	[118-119,132,187-189, 227]
$MSRA^{+/0}$	Normal	Reduced reperfusion injury, sensitive to oxidative stress.	[112]
$PCat^{+/0}$	Normal	Protects against hypertension in angiotensin-II mice. Cardiac contractility improves in type 1 &2 diabetes.	[214-216]
$NCat^{+/0}$	Normal	Carcinomas sensitized to bleomycin and DNA damage to paraquat. Reduced DNA damage by hydrogen peroxide.	[217-218]
$MCat^{+/0}$	↑21%	Invasive breast tumors, protection from cardiac myopathy in mutator mice and anti-retrovirals, as well as aging-dependent pathologies.	[207-213]
$ECSOD^{+/0}$	nd	Reduces cardiovascular abnormalities after reperfusion injury, and hyperoxia damage to lungs.	[134,232-234]
$MnSOD^{+/0}$	Normal	No benefit from lung irradiation or muscle atrophy. Improves diabetic cardiomyopathy.	[14,117,228-229]
$CuZnSOD^{+/0}$	Normal	No effect on diaphragm fatigue or recovery.	[14,122]

Antioxidants with changes in life- or healthspan are presented. However, many antioxidant transgenic models have not yet been generated, which includes methionine sulfoxide reductase B, peroxiredoxin-1, -2, -5, glutaredoxin-3, -4, -5, -6, -7, -8, thioredoxin reductase-1, -2, -3, thioredoxin 2, glutathione peroxidase-2, -3, -5, -6, -7, -8 and nicatinamide nucleotide transhydrogenase.nd not determined