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Oxidative stress in vascular senescence: lessons from successfully aging species

Zoltan Ungvari[1], Rochelle Buffenstein[2], Steven N. Austad[2], Andrej Podlutsky[2], Gabor Kaley[1], Anna Csiszar[1]

[1]Department of Physiology, New York Medical College, Valhalla, New York 10595, USA, [2]The Sam and Ann Barshop Institute for Longevity and Aging Studies, The University of Texas Health Science Center, San Antonio, Texas 78245

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1. ABSTRACT

Cardiovascular disease is a main cause of morbidity and a leading cause of death of elderly Americans. Studies identifying the pathophysiological mechanisms underlying cardiovascular aging hold promise to develop treatments to delay/prevent coronary artery disease and stroke in the elderly. Evidence supporting the roles of oxidative stress and inflammation in the cardiovascular aging process is presented in detail in this review. Mammalian lifespan ranges hundred-fold and we propose that long-living species may be useful models for successful cardiovascular aging in humans. Comparative studies exploiting the large differences in maximum lifespan potential and cardiovascular aging patterns may be particularly relevant. Comparisons of mechanisms related to oxidative stress, oxidative stress resistance and redox signaling between long-living species and shorter-living ones may elucidate key mechanisms for delaying cardiovascular aging. We discuss the potential use of three long-lived but mouse-sized mammalian species, the naked mole-rat (*Heterocephalus glaber*), the white-footed mouse (*Peromyscus leucopus*) and the little brown bat (*Myotis lucifugus*) to test predictions of the oxidative stress theory of aging and elucidate mechanisms by which cardiovascular aging can be delayed.

2. INTRODUCTION

Epidemiological studies showed that even "healthy" aging is a major independent risk factor for cardiovascular disease. Cardiovascular disease is a main cause of morbidity and a leading cause of death of elderly Americans, responsible for approximately 50% of deaths of those over 65 years of age. There are over 35 million people in the United States (about 13 percent of the total population) 65 years of age or older and their number will double in the next two decades. The increasing number of older persons will likely lead to increased incidence of aging-induced cardiovascular disease imposing a significant burden on the country's health care system. Understanding of the mechanisms underlying cardiovascular aging and aging-induced vascular pathophysiological alterations may hold promise in addressing this upcoming burden.

The mechanisms by which endothelial oxidative stress and vascular inflammatory processes act as potent pro-atherogenic stimuli have been the subject of intense study. This review focuses on the emerging evidence that reactive oxygen species (ROS) and activation of inflammatory pathways play a central role in cardiovascular aging, and discusses the non-traditional

experimental use of animal models of longevity, which have the potential to elucidate critical mechanisms for promotion of cardiovascular health in the elderly.

3. VASCULAR OXIDATIVE STRESS IN AGING

Harman originally proposed the free radical theory of aging half a century ago (1), yet the relationship between oxidative stress and aging is still much debated. The mitochondrial theory of aging, put forward by Harman (2) postulates that mitochondria are the main source of ROS in aged cells. According to this theory, mitochondria-derived H_2O_2 diffuses readily through cellular membranes and contribute to a variety of macromolecular oxidative modifications. This original working concept invoked accumulation of such oxidative damage of proteins, lipids and DNA as the primary causal factor in the aging process (2). Antioxidants may neutralize ROS, thereby attenuating damage accrual. Indeed, in lower organisms overexpression of antioxidant enzymes and/or treatment with antioxidants seem to extend lifespan (3). There is considerable evidence that aging in mammals is associated with oxidative stress and oxidative macromolecular damage accrues with age in virtually every tissue studied (4-12). Yet, genetic knockout mice for major cellular antioxidant enzymes show a relatively mild phenotype despite the significant increases in ROS levels, and higher levels of oxidative damage in all tissues, often with no major change in maximum lifespan potential (MLSP) (13-15). Although there are reports suggesting that overexpression of catalase may increase lifespan (16), in many studies transgenic mice overexpressing antioxidant enzymes involved in scavenging of $O_2^{\cdot-}$ and H_2O_2 do not exhibit an extended longevity phenotype (17, 18). Furthermore dietary supplementation with antioxidants does not appear to retard age-related declines in mammals. Indeed despite the many years of research focusing on potential antioxidant therapy as well as the development of a billion dollar antioxidant industry, there is little or no well-authenticated clinical studies that unequivocally demonstrate a benefit (19). Clearly aging is a multifacetted process of which the role of antioxidants is only one player.

In contrast, the general concept that oxidative stress is involved in many age-related diseases, including atherosclerosis, hypertension, diabetic vasculopathy and Alzheimer's disease, appears robust. Considerable evidence has been published that increased production of reactive oxygen species underlies endothelial dysfunction in aging. Recently we and others showed that in small coronary arteries (7), mesenteric arteries (10) of aged rats there is an increased $O_2^{\cdot-}$ and H_2O_2 production. Similarly, increased ROS production has also been reported in the aorta and carotid arteries of aged rats and mice (9, 11, 12, 20, 21). Oxidative stress-induced endothelial dysfunction is also present in humans (22, 23). Ascorbic acid infused at concentrations known to scavenge reactive oxygen species restores resting femoral artery blood flow in healthy older adult males by increasing vascular conductance (22). Aging-induced vascular oxidative stress seems to be associated with a pro-oxidant shift in vascular phenotype, including an increased expression of iNOS (12, 24) and increased activity of NAD (P)H oxidases (7, 11, 23, 25) and/or other oxidase mechanisms (26) and a down-regulation of antioxidants, such as ecSOD (10).

Over the last two decades, major refinements have emerged in our understanding of how ROS affect vascular homeostasis. It has been established that nitric oxide (NO) is a crucial factor for the health and function of endothelial cells. One of the consequences of increased oxidative stress in aging is a functional inactivation of NO by high concentrations of $O_2^{\cdot-}$ resulting in an enhanced $ONOO^-$ formation (7, 10, 12, 25). Cardiovascular aging is characterized by a gradual decline in NO bioavailability and, consequently, a deterioration of endothelial function (Figure 1) and myocardial performance both in experimental animals and in humans (8, 24, 27-30), which begins to accelerate after mid-life. It is generally accepted that tonic release of NO from the endothelium exerts vasculoprotective and cardioprotective effects, such as maintenance of normal coronary blood flow, inhibition of platelet aggregation and inflammatory cell adhesion to endothelial cells and disruption of pro-inflammatory cytokine-induced signaling pathways. The severe impairment of NO bioavailability in aging, also aggravated by an age-related decline in eNOS expression (7, 31-34) and/or a decreased intracellular L-arginine availability (35), limits cardiac blood supply and alters myocardial O_2 consumption and cardiac contractility (25). Although resting myocardial blood flow is slightly higher in older subjects (due to the increased systolic blood pressure in the elderly), hyperemic myocardial blood flow declines over 55 years of age, which is likely a consequence of aging-induced microvascular endothelial dysfunction. As a result coronary flow reserve is significantly reduced in older subjects. Recent studies also suggest that decreased endothelial NO production in aging enhances apoptosis of endothelial cells (34, 36). There is also an emerging view that ROS, in addition to inactivating NO and causing oxidative damage, play important signaling roles in the vascular wall as well. Importantly, oxidative/nitrosative stress and the consequent activation of numerous downstream effector pathways are thought to be implicated in the inflammatory process in the aged vasculature.

4. VASCULAR INFLAMMATION IN AGING

In the past decades atherosclerosis has been recognized as an inflammatory disease (37). Recent studies have shown that even in "healthy aging" there is a pro-inflammatory shift in vascular (7, 36, 38) and cardiac (39) gene expression profile (including an up-regulation of TNFα, IL-6 and iNOS; Figures 2 and 3). There is growing evidence that high levels of inflammatory cytokines contribute to a pro-inflammatory microenvironment that facilitates the development of cardiac and vascular dysfunction in aging. In particular, it has become established that vascular aging is associated with dysregulation of tumor necrosis factor (TNF)-alpha expression (7, 36, 38, 40, 41). TNFα is a master regulator of vascular proatherogenic phenotypic changes, and it has been linked to endothelial dysfunction and apoptosis. Increased production of pro-inflammatory cytokines is also

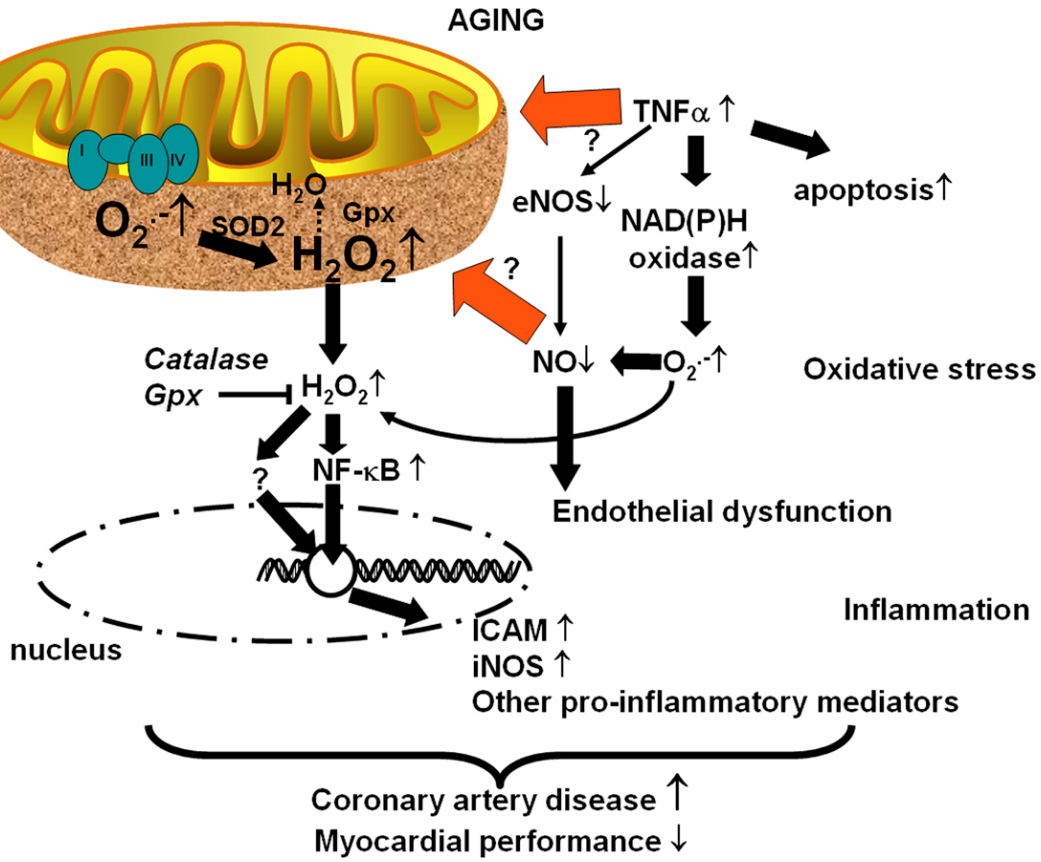

Figure 1. Proposed scheme for the link between oxidative stress and vascular inflammation in aging: in aged endothelial cells increased levels of $O_2^{\cdot-}$ generated by the electron transport chain are dismutated to H_2O_2, which can penetrate the mitochondrial membranes increasing cytoplasmic H_2O_2 levels. H_2O_2 contributes to the activation of NF-κB, resulting in a pro-inflammatory shift in endothelial gene expression profile. Increased $O_2^{\cdot-}$ production by the NAD (P)H oxidase (stimulated, at least in part, by elevated TNFα levels in the vascular wall) and/or down-regulation of eNOS is responsible for the impaired bioavailability of NO and endothelial vasodilator dysfunction in aged arteries. The model predicts that up-regulation of TNFα and/or impaired NO bioavailiability may also contribute to the development of mitochondrial oxidative stress in aging. Increased TNFα levels also promote endothelial apoptotic cell death, which together with the increased oxidative stress and vascular inflammation increase the risk for the development of coronary artery disease.

associated with premature vascular aging (e.g. in metabolic diseases) and is characteristic of age-associated vascular diseases (e.g. atherosclerosis, Alzheimer disease) (42, 43). Plasma levels of TNFα (44) increase in aging and correlate with morbidity and mortality in the elderly (45). It is significant that chronic anti-TNFα treatment (with etanercept, which binds and inactivates TNFα) seems to exert multifaceted vasculoprotective effects in aged rats (40, 46, 47). Among these, etanercept treatment significantly improves endothelial function and decreases vascular NAD (P)H oxidase activity and expression (40, 46). In aged vessels up-regulated expression of various inflammatory markers also can be decreased by etanercept treatment (40).

In the last decade it has been established that aging is associated with enhanced endothelial apoptotic cell death (34, 36, 40, 48-50). TNFα has been recognized as one of the most potent inducers of programmed cell death in endothelial cells (51) and cardiac myocytes (52) and the facilitating role of TNFα in this context in aging is undeniable (36, 40). Preventing TNFα from binding its membrane-bound receptor by etanercept treatment significantly reduced endothelial apoptosis in aged rats (40). In line with the aforementioned findings, in carotid arteries of young animals, recombinant TNFα can elicit endothelial dysfunction, oxidative stress, and increased apoptosis and proinflammatory gene expression, mimicking many of the symptoms of vascular aging (40). Previous studies suggest that endothelial dysfunction and endothelial cell injury due to the activation of the cellular apoptotic pathways are an initial step in the development of CAD (53) and heart failure. Importantly, initial clinical studies demonstrated beneficial effects of anti-TNFα therapy on cardiac performance (54, 55) in humans with heart failure. Also, serum of patients with heart failure induced apoptosis in cultured endothelial cells, an effect that was antagonized by an anti-TNFα neutralizing antibody (56). Preliminary studies raised the possibility that anti-TNFα treatments may improve cardiac function in aged F344 rats (Figure 3).

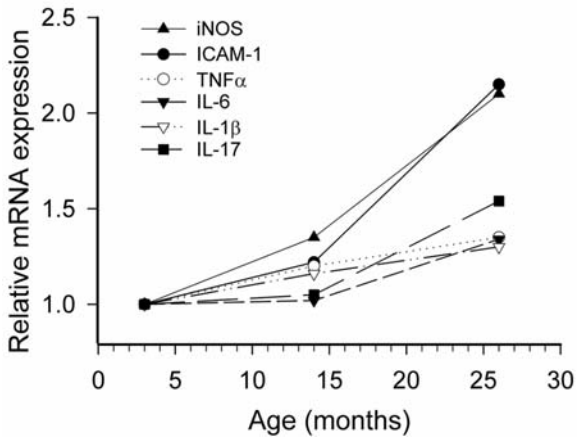

Figure 2. Results from gene expression profiling of vascular aging. We have used high density oligonucleotide arrays (Affymetrix) to identify functional classes that may uncover biological processes that play a role in vascular aging. In carotid arteries of C57 mice (n=4-9 animals in each age group) we detected age-related up-regulation of various inflammatory cytokines and other inflammatory genes, such as ICAM-1 and iNOS (please note that expression of low abundance genes, such as cytokines, are under-estimated by this microarray method due to the low signal-to-noise ratio). Similar age-related alterations in vascular inflammatory gene expression have been found previously in F344 rats (7, 21, 36, 38, 40). These observations support a role for inflammation in vascular aging in mammals.

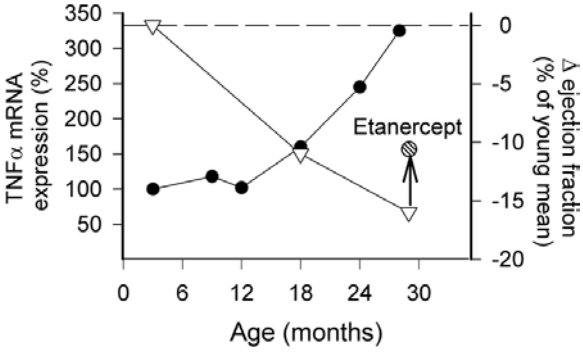

Figure 3. Age-dependent increases in vascular TNFα mRNA expression in F344 rats (filled circles). mRNA expression was quantified by QRT-PCR. Figure is redrawn based on data presented in reference (40). The time course of TNFα induction coincides with the age-dependent decline in cardiac performance (open triangles; ejection fraction was measured by echocardiography as described (119)). Chronic (1 month) etanercept treatment tended to improve cardiac contractility in aged rats (arrow).

Thus, further studies are definitely needed to elucidate whether anti-TNFα treatments exert anti-aging vasculoprotective effects in humans. Inhibition of the endocannabinoid anandamide metabolizing enzyme, the fatty acid amide hydrolase (FAAH), is also emerging as a promising novel approach for the treatment of various inflammatory disorders. It is significant that the aging-associated decline in cardiac function and increased myocardial gene expression of TNFα, iNOS and $gp91^{phox}$, increased nitrotyrosine formation, enhanced apoptotic cell death observed in aged $FAAH^{+/+}$ mice, are largely attenuated in $FAAH^{-/-}$ mice (41). In addition, targeting of cannabinoid-2 (CB2) receptors with selective agonists *in vitro* were shown to disrupt TNFα-induced proinflammatory signaling pathways in endothelial cells (57). Thus, CB2 receptor antagonists may also offer a novel therapeutic target to inhibit TNFα-induced cardiovascular inflammation in aging.

Recent studies have uncovered an important cross-talk between inflammatory cytokines, generation of ROS and reactive nitrogen species (RNS) and pro-inflammatory gene expression in the pathogenesis of cardiovascular aging. NF-κB is a redox-sensitive transcription factor that is expressed by both endothelial and smooth muscle cells. Activation of NF-κB is thought to induce the transcription of a large range of genes implicated in inflammation, including cytokines, chemokines and adhesion molecules (58-60). It is also generally believed that chronic activation of NF-κB predisposes arteries to atherosclerosis (61). Numerous studies demonstrated that increased levels of ROS may activate NF-κB in endothelial, smooth muscle cells and other cell types, leading to the up-regulation of adhesion molecules, iNOS and other inflammatory mediators. Of note, recent studies have demonstrated that NF-κB binding increases in aging (21, 23, 62), which is likely responsible for the increased expression of adhesion molecules and iNOS found in aged coronary vessels (7), carotid arteries and aortas (21, 63). NF-κB activation and chronic inflammation seems to be a generalized phenomenon in aging, because increases in NF-κB activity have been observed in the aged rat skeletal muscle, liver, brain and cardiac muscle as well (62, 64-66). The finding that scavenging of H_2O_2 attenuated NF-κB activation in aged vessels (21), suggests a role for H_2O_2 in regulation of endothelial NF-κB activity in aging. This view is in line with the finding that exogenous H_2O_2 substantially increased NF-κB activation in vessels of young rats, mimicking the aging phenotype (21). Several lines of evidence suggest that mitochondria are a major source of H_2O_2 in aged blood vessels (21). These observations suggest that age-related decline in mitochondrial function is, at least in part, responsible for vascular inflammation in aging (21). Local leukocyte recruitment into the vessel wall is an early step in atherogenesis and is controlled by the expression of cell adhesion molecules. It is significant that inhibition of mitochondrial ROS production was shown *in vitro* to decrease endothelial ICAM-1 expression and attenuate monocyte adhesiveness to the endothelium in aged rat arteries (21). In aging mice that overexpress human catalase in the mitochondria (MCAT) cardiac pathology was delayed, oxidative damage was reduced, H_2O_2 production and H_2O_2-induced aconitase inactivation were attenuated, and the development of mitochondrial deletions was reduced (67). It is yet to be seen, whether inflammatory gene expression is also attenuated in the cardiovascular system of MCAT mice. At present it is

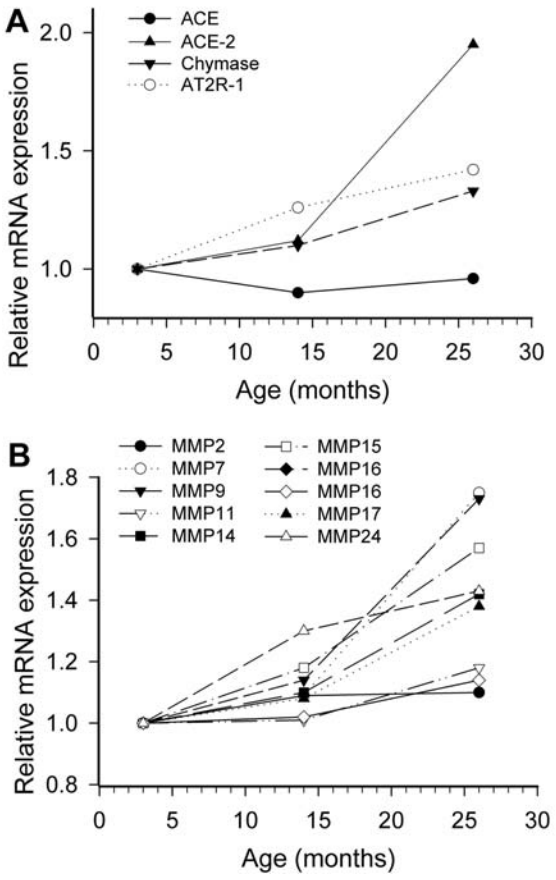

Figure 4. Aging is associated with widespread changes at the gene expression level in carotid arteries of C57 mice. Panel A shows gene expression changes suggesting up-regulation of tissue renin-angiotensin system (ACE: angiotensin converting enzyme, AT2R-1: angiotensin II receptor-1), whereas Panel B depicts gene expression pattern characteristic of a generalized induction of matrix metalloproteases (MMP) in the vascular wall. These observations are completely in line with the findings of Lakatta's group (72-75) in non-human primates and support a role for RAS in vascular aging.

unknown whether systemic inhibition of NAD (P)H oxidase activity or administration of mitochondria-targeted antioxidants would affect progression of cardiovascular diseases in elderly patients. Although previous observational and epidemiologic studies have suggested that dietary supplementation with antioxidants or a diet rich in antioxidants might interfere with formation of atherosclerotic lesions, recent large randomized clinical trials have shown no significant benefit when vitamin E was given to patients after myocardial infarction or in those with vascular disease or diabetics with a high-risk CAD profile (68-71). It is likely that vitamin E is not the best antioxidant agent to block cellular free radical signaling (it goes directly in the lipid membranes and protects them from peroxidation, whereas ROS likely act as signaling molecules/second messengers primarily in cytosolic/sub-sarcolemmal microdomains). Drugs that directly inhibit ROS producing enzymes (such as NAD (P)H oxidases) are expected to have superior efficacy in disrupting ROS-induced cellular signaling pathways.

Landmark studies by Dr. Edward Lakatta's laboratory called attention to the association of an up-regulated tissue renin-angiotensin system (RAS) with intimal thickening and remodeling in large arteries of aged animals and humans (72-75). It has been demonstrated that angiotensin-converting enzyme (ACE), angiotensin II, angiotensin II receptor type 1, matrix metalloproteinases (MMP) 2/9 and monocyte chemoattractant protein-1 increase within the wall of these arteries with aging (72-75). Our own data obtained in mouse carotid arteries (Figure 4) are in agreement with the findings in human and monkey arteries. The available data suggest that up-regulation of RAS contributes to chronic vascular inflammation (and perhaps oxidative stress) in aging, enhancing vascular response to injury and rendering the vascular wall susceptible for the development of atherosclerosis. The MMPs can act together to degrade the major components of the vascular extracellular matrix. MMP activation is likely to contribute to intimal growth and vessel wall remodeling in response to injury, most notably by promoting migration of vascular smooth muscle cells. A higher level of MMP activation in aged arteries, especially associated with inflammation, could contribute to pathological matrix destruction and plaque rupture.

5. NOVEL ANIMAL MODELS OF SUCCESSFUL VASCULAR AGING: TESTING PREDICTIONS OF THE OXIDATIVE STRESS HYPOTHESIS OF AGING

Mammals have the same cell structure and biochemistry, yet their lifespan ranges hundred-fold. The house mouse (*M. musculus)* is amongst the fastest aging mammals (maximal lifespan potential (MLSP): ~3.5 years; human MLSP: 122 years) and therefore a popular subject of cardiovascular aging studies. The mouse genome is published and the animal's short life span enables longitudinal studies and experimental manipulations. Yet, mice are primarily chosen for convenience, rather than for specific features pertinent to human aging. Long-living species may be useful models for human aging and comparative studies exploiting the large differences in MLSP and cardiovascular aging patterns may be particularly relevant. Comparisons of mechanisms related to oxidative stress, oxidative stress resistance and redox signaling between long-living species and shorter-living ones may elucidate key mechanisms for delaying cardiovascular aging.

The oxidative stress theory of aging predicts that long-lived, successfully aging animals utilize one or more of the following three potential strategies to delay/limit oxidative stress-induced cellular damage (Figure 5): 1) lower initial generation of reactive oxygen species (ROS, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2)) at young ages, so that it takes longer to reach the critical threshold (beyond which oxidative damage significantly impairs cellular function) even at the same rate of aging, 2)

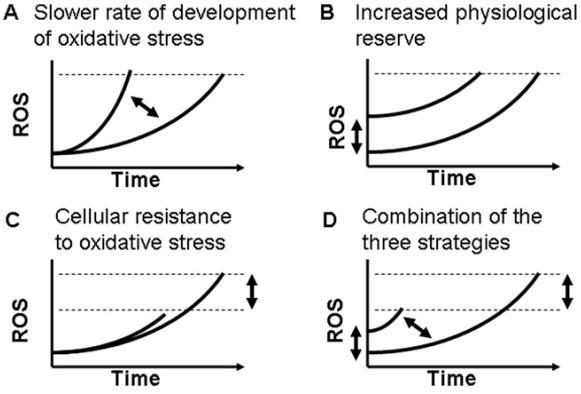

Figure 5. Schematic representation of potential strategies by which long-lived, successfully aging animals can delay/limit oxidative stress-induced damage in the cardiovascular system and in other organs. Panel A: Slower rate of age-related increases in ROS generation (increasing the time to reach a threshold, beyond which oxidative damage significantly impairs cellular function). Panel B: Lower initial ROS generation at young ages, so that it takes longer to reach the critical threshold even at the same rate of aging. Panel C: Increased tolerance for age-related increases in ROS production. Panel D: Long-lived animals may utilize a combination of all three strategies. Non-linear/exponential characteristics of age-related ROS increases are based on $O_2^{\cdot-}$ production in blood vessels of F344 rats (20) as well as on DNA oxidation in liver, brain, kidney, heart, and skeletal muscle of the same strain (6).

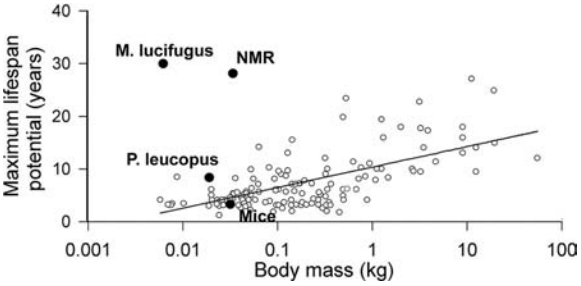

Figure 6. The relationship between body mass and maximum species lifespan potential (MLSP) for rodents (data points were taken from references (76, 92); NMR: naked mole rat). Bats (such as *M. lucifugus*, which is also represented in this graph) are extremely long-lived for their small size. The line represents the predicted MLSP, which is based on the allometric equation of Austad and Fisher (120) (predicted MLSP=10.67 x $M_{kg}^{0.189}$)). Longevity quotient (given in Table 1.) is calculated from the ratio of maximum longevity to the predicted MLSP.

increased tolerance for increases in ROS production (including superior antioxidant defense and/or damage repair mechanisms) and 3) slower rate of age-related increases in ROS generation (increasing the time to reach a critical threshold).

Most comparative studies on lifespan and physiology have not taken into account the possible confounding effects of body mass and phylogeny. As shown in Figure 6, there is general correlation between body mass and longevity: short-lived species tend to be relatively small, while the long-lived species are larger. The effects of body mass can be circumvented by comparing animals of similar body mass but differing lifespans. In the aging field, this has been achieved by comparing birds such as pigeons (MLSP: ~35 years) with mammals with similar body masses such as rats. However, in these cases there are issues associated with phylogeny: it may be that certain traits (such as macromolecular damage) are low in all birds for reasons unrelated to their longevity. In the present review we will discuss the potential use of three long-lived but mouse-sized mammalian species, the naked mole-rat (*Heterocephalus glaber*), the white-footed mouse (*Peromyscus leucopus*) and the little brown bat (*Myotis lucifugus*) to test predictions of the oxidative stress theory of aging and elucidate mechanisms by which cardiovascular aging can be delayed. These species have been specifically chosen because relative to that predicted by body size they are longer-living. Naked mole-rats and bats live three times longer than *P. leucopus* while *P. leucopus* lives twice as long as do mice. These patterns of disparate longevity may enable researchers to test if oxidative stress features correlate with maximum lifespan or if more simply longer-living species have more efficient protection against damage accrual.

5.1. Naked mole-rats

The evolutionary theory of aging predicts that life span should increase in response to a decreased level of mortality caused by extrinsic sources (i.e. predation). The naked mole-rats (Rodentia:- Bathyergidae; *Heterocephalus glaber*) are mouse-sized East-African rodents that lead a strictly subterranean existence. They show exceptional longevity and have a longevity quotient (LQ; the ratio of actual MLSP to that predicted by body mass) similar to that of humans (Table 1.). The use of the naked mole-rat as a model for biogerontological research is a subject of a recent review (76).

Vascular endothelial cells of long-lived species in general tend to produce less ROS than shorter-living ones even at young ages (see below). The naked mole-rat seems to be an exception from this rule, as previous studies in these animals could not demonstrate a positive correlation between MLSP, endothelial H_2O_2 generation and tissue glutathione peroxidase activity (77, 78). Interestingly, naked mole-rats in various tissues exhibit similar, or sometimes even greater, levels of accrued oxidative damage to lipids, DNA and proteins than age-matched mice (76, 78-81). At present it is unclear what the cause is for the relatively high rate of macromolecular oxidative damage in naked mole-rat cells. One possible explanation is that naked mole-rats in the wild are living in a hypoxic environment underground, whereas in the laboratory they are exposed to 21% oxygen, which may be cause a relative hyperoxaemia leading to a higher basal rate of ROS production.

The mitochondrial oxidative stress hypothesis of aging predicts that if mitochondrial ROS production is

Table 1. Characteristics of long-lived animal models

	M. musculus	*H. glaber*	*P. leucopus*	*M. lucifugus*
body weight (g)	~28	~35	~21	~*8*
MLSP (years)	~3.5	>28.3	~8	>30
LQ	0.64	5.00	1.55	~7
Baseline endothelial H_2O_2 production vs. mice	N/A	→	↓	↓
Baseline endothelial $O_2^{\cdot-}$ production vs. mice	N/A	→	↓	↓
Stimulated (oxLDL, high glucose) endothelial ROS production	↑↑↑	?	↑	?
NAD (P)H oxidase expression/activity vs. mice	N/A	↓	↓	?
eNOS expression/activity vs. mice	N/A	?	↑	?
Expression of cellular antioxidant systems (Gpx-1, SOD isoforms, catalase) vs. mice	N/A	→↓	↑	?
Mitochondrial ROS production vs. mice	N/A	↓	↓	↓
Oxidative stress-induced endothelial apoptosis	↑↑↑	↑→	↑→	↑
Aging-induced cellular oxidative stress	↑↑↑	↑→	?	?
Aging-induced vascular apoptotic cell death	↑↑↑	↑→	?	?
Aging-induced endothelial dysfunction	↑↑↑	→	?	?
Aging-induced inflammatory gene expression	↑↑↑	?	?	?
Oxidative stress-induced DNA damage	↑↑↑	↑	↑↑	↑
Cellular DNA repair capacity vs. mice	N/A	↑↑	↑	↑↑

Longevity quotient were calculated from the ratio of maximum longevity to the predicted maximum lifespan potential (based on the allometric equation of Austad and Fisher (120) for nonflying eutherian mammals (predicted longevity=10.67 x $M_{kg}^{0.189}$) and/or the equation of Jurgens and Prothero for all mammals (121, 122)). ↓: smaller/less than in mice, ↑: greater/more than in mice

important in determining the rate of aging, then long-lived animals should produce less. Recent data seem to support this premise: the rate of ROS production in cardiac mitochondria in naked mole-rats is less than those from mouse (82). Similar conclusions were reached also by studies showing lower H_2O_2 generation in mitochondria isolated from the heart of the long-lived damara mole-rats relative to shorter lived similar sized guinea pigs (82) and white footed mouse (83) (*Peromyscus leucopus*; MLSP: 8 years; see below), small brown bats (82, 84) (*Myotis lucifugus,* MLSP: 31 years, see below) and avian species (canary, MLSP: 24 years (85); parakeet, MLSP: 21 years (85); pigeons, MLSP: 35 years (86)). There is also data showing that oxidative damage to mtDNA is inversely related to maximum life span in the heart and brain of mammals (87, 88).

In spite of the similar endothelial ROS production at young ages, our recent studies demonstrated that in long-lived naked mole-rats age-related development of oxidative stress and endothelial dysfunction (Figure 7A,B) is substantially delayed. Also, NAD (P)H oxidase expression (which shows marked up-regulation in aged rats and mice) does not change with age in this extremely long-lived species (20). In addition, we could not detect significant age-related changes over a more than two decade life interval in mitochondrial gene expression in the hearts of naked mole-rats (up to 24 years of age) (20).

Recent studies revealed that in most species advanced age promotes apoptotic cell death in various tissues, including peripheral arteries (36, 48) and the heart (89-91). It is significant that long-living naked mole-rats are protected from age-dependent increases in endothelial apoptosis (20) (Figure 7C). Similar findings were obtained recently in long-lived Damara mole-rats (*Cryptomys damarensis*, MLSP: 16 years) and a bat species (see below), in which a negative correlation exists between MLSP and H_2O_2 –induced apoptotic cell death in the blood vessels (77). Because vascular tissue from naked mole-rats is resistant to the apoptotic effects of in vitro administered H_2O_2 (92), we hypothesize that delayed age-related development of oxidative stress together with the remarkable cellular resistance to oxidative stress will preserve cardiovascular function throughout the lifespan of these animals. Interestingly, there is also an increased resistance to oxidative challenge in cultured fibroblasts from certain long-lived mouse models (such the Ames and Snell dwarf mice (93-95)), long-lived rodents (96) and long-lived birds (budgerigar, *Melopsittacus Undulatus*, MLSP: ~20 years) (97).

5.2. Peromyscus leucopus

The white-footed mouse (*Peromyscus leucopus*) despite its close resemblance to the house mouse (*Mus musculus*) has an unusually long lifespan for its size (Table 1.). In the wild *M. musculus* and *P. leucopus* (which share a common ancestor 20-25 million years ago (98)) are reported to have similar short lifespan (although this observation is based on only one, quite old, study of Peromyscus) because of the high risk for extrinsic mortality due to predation and the lack of food in the winter. However, in captivity *P. leucopus* has more than 2-fold greater life span than *M. musculus* (the record longevity for *P. leucopus* in captivity is 7.9 years (99, 100)). Previous data showed that in aged *P. leucopus* the hypothalamic-pituitary-ovarian axis remains intact and fertility is maintained (100, 101), rate of accumulation of DNA damage (in liver and kidney cells) is delayed (102) and that aged *P. leucopus* (up to 66 months of age) did not develop visible tumors (100). Because of these considerations *P. leucopus* seems to be a useful model of successful aging in small muroid rodents.

In a series of studies we are currently comparing endothelial function, vascular ROS generation and cellular oxidative stress resistance in *P. leucopus* and mice. We have recently reported that in arteries of *P. leucopus* there is an attenuated production of ROS ($O_2^{\cdot-}$, H_2O_2) from NAD (P)H oxidase (83). The differences in NAD (P)H oxidase activity in short- and long-lived species is significant, because up-regulation of NAD (P)H oxidase was shown to

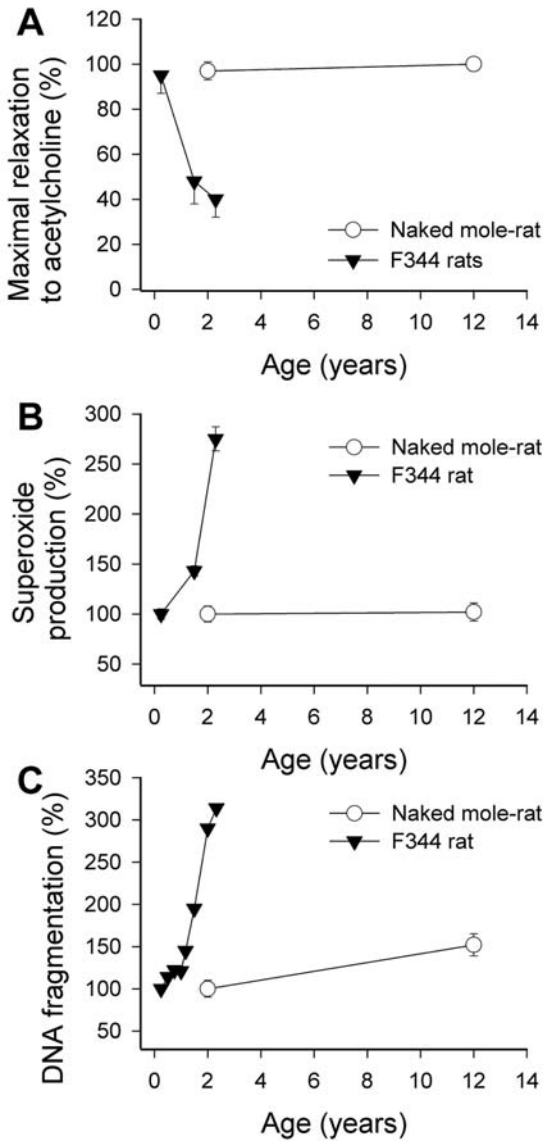

Figure 7. Decreased age-related oxidative stress in long-lived *H. glaber*. **A:** Maximal relaxation induced by acetylcholine in carotid arteries of naked mole-rats and F344 rats as a function of chronological age. Panel B: $O_2^{\cdot-}$ production (assessed by the ethidium bromide fluorescence method) in arteries of F344 rats and naked mole-rats as a function of chronological age. Panel C: Apoptotic cell death (assessed by increases in DNA fragmentation) in arteries of naked mole rats and F344 rats as a function of chronological age. Figure is redrawn based on data from reference (20).

underlie increased $O_2^{\cdot-}$ generation in vessels of aged rats (7, 25, 103) and contribute to vascular pathophysiological alterations in hypertension and metabolic diseases (diabetes, hyperhomocysteinemia and hypercholesterolemia), which many investigators consider "accelerated vascular aging" (based on similarities of the gene expression profile in senescent vessels).

In addition to the NAD (P)H oxidase, mitochondria can also contribute significantly to vascular ROS production (21). The findings showing that the rate of ROS production both vascular and cardiac mitochondria in *P. leucopus* is substantially less than those from mouse (83, 84, 104) agree with the prediction of the oxidative stress hypothesis of aging. In *P. leucopus* there is also a higher Gpx-1 and catalase content and a more abundant expression of eNOS associated with increased endothelial NO production in large arteries (83). In addition, endothelial cells of the longer-living *P. leucopus* are substantially more tolerant of oxidative stress (induced by oxLDL or H_2O_2 treatment) than those of shorter-living mice (83) (Figure 8). Previous studies also have shown that brain and heart of *P. leucopus* have higher activities of catalase and glutathione peroxidase (104) and lower levels of protein oxidative damage as well as lower susceptibility to oxidative damage in response to experimental oxidative stress (104) than those of mice. Previous studies demonstrated that inhibition of glutathione peroxidase in various cell types, including endothelial cells, enhances oxidative stress-induced apoptosis (49, 105, 106). Also, there is a positive correlation between glutathione peroxidase activity and oxidative stress resistance in human cell lines (107). These findings suggest that the greater cellular glutathione peroxidase content may contribute to the superior oxidative stress resistance in *P. leucopus.* Inhibition of hemeoxygenase-1 (HO-1) also can enhance oxidative stress-induced apoptosis in endothelial cells (49, 108, 109). Because expression of HO-1 is also greater in vessels of *P. leucopus* than in mouse arteries (83), we posit that HO-1 may also contribute to cellular resistance to oxidative damage in *P. leucopus* cells.

The estimation of cellular resistance to oxidative stress by assessing extent of DNA damage and repair after oxidant challenge is an important aspect of studies on successful aging. The single cell gel electrophoresis ("comet") assay is a new, simple and sensitive method of evaluating DNA damage and repair at the level of individual cells. Previous research raised the possibility that there is a positive correlation between maximum longevity and the rate or fidelity of DNA repair. We are currently comparing relative rate of DNA repair in fibroblast cell lines from various long- and short lived species (Podlutsky and Austad; unpublished data 2007). Our results so far indicate that longer-living species, including *P. leucopus* (83), non-human primates and bats, exhibit less H_2O_2 - induced DNA damage than mouse cells. In addition, fibroblasts of long-lived species tend to repair DNA damage much faster then short-lived ones. To investigate whether a similar relationship holds true for endothelial cells from these species requires further research. Also, the mechanisms responsible for the superior cellular protection of long-lived species against oxidative stress-induced DNA damage (i.e. relative contribution of better antioxidant defenses, superior DNA repair systems, and/or a lower vulnerability of the DNA due to chromatin packaging) needs to be elucidated.

5.3. Bats

On average, the life span of bats (Order: Chiroptera) is approximately 3 times greater than a non

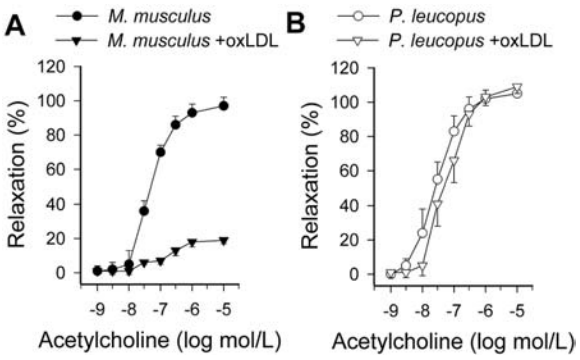

Figure 8. Oxidative stress resistance in long-lived *P. leucopus*. **A:** Relaxation to acetylcholine is significantly impaired in oxLDL-treated mouse aortic segments (24 h, in organoid culture), whereas it is preserved in oxLDL-treated vessels of *P. leucopus* (B). (Data are mean±S.E.M., n=5 for each group). Figure is redrawn based on data from reference (83).

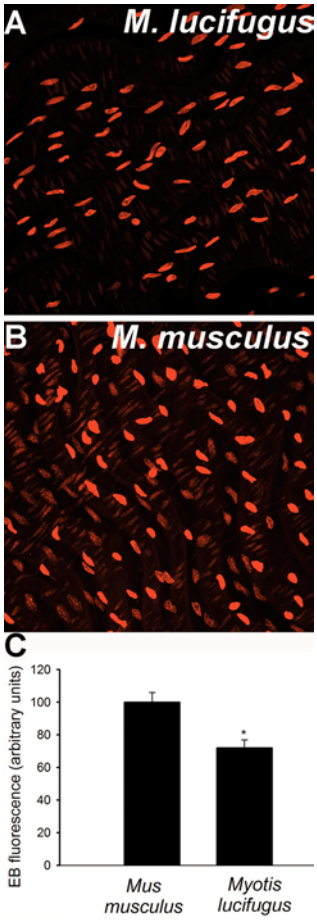

Figure 9. Decreased ROS production in long-lived *M. lucifugus*. Representative confocal images showing dihydroethidine staining (red fluorescence) in endothelial cells of aortas from *M. lucifugus* (A) and mice (B; original magnification: 40x). Bar graphs (C) are summary data for ethidium bromide (EB) fluorescence. Data are mean ±S.E.M. (n=5 animals in each group) *P<0.05.

flying placental mammal of similar size (110, 111). Recently a new longevity record of 41 years has been reported for a free-living bat (Myotis brandtii) (110). Thirteen species in the genus Myotis, ranging in size from 7 to 25 grams, have been documented to live at least 20 years in the wild (110). The exceptional longevity of bats (Figure 6), which is unusual for mammals of such a small size and a high metabolic rate, renders them an interesting animal model of slow aging. Bat longevity results from neither low basal metabolic rate (it is telling that the highest recorded heart rate for *M. lucifugus* is 1368 bpm), nor large relative brain size. These data directly conflict with predictions of both "rate of living" and brain-size mediated theories of aging (110, 111). However, bats comply with the predictions of the evolutionary theory of aging, that posits exceptionally long life spans among mammals with reduced environmental vulnerability (bats are able to escape extrinsic mortality through flight and nocturnal life) (111). In addition, bats have other life history traits that are usually characteristic of larger long-lived mammals (e.g. few and large offspring, slow growth rates) (112). In our ongoing studies we are comparing physiological variables related to longevity and cardiovascular health in mice and a common bat species, the little brown bat (Myotis lucifugus). M. lucifugus lives approximately 6 to 7 years and often lives well beyond 10 years (the oldest individual captured in the wild was a 31 year-old male) (113). The range of the species covers most of North America (113), thus these bats can be relatively easily obtained for laboratory studies.

Our data suggest that endothelial cells in bat arteries produce significantly less O2.- (Figure 9) and H2O2 (Figure 10) than mouse vessels. It is still unknown whether this is due to a lower constitutive activity of NAD (P)H oxidases or more efficient mitochondria in this species. The findings that mitochondria from the heart of *M. lucifugus* produce at least 50% less H_2O_2 per unit of oxygen consumed compared to those from short-lived rodents (82, 84, 114) warrant further studies on the role of mitochondria in vascular ROS production as well. The antioxidant capacity in the cardiovascular system of bats has not yet been systematically evaluated. There is some data available that cardiac SOD activity may not differ between bats and shorter-living species (84, 114). In various bat species the contents of alpha-tocopherol and beta-carotene found in the heart, liver, kidneys, and pectoral muscles were reported to be one to two orders of magnitude higher than those usually found in rat and mouse tissues (115). Our findings (Figure 11) suggest that endothelial cells of M. lucifugus are more resistant to oxidative stress than those of mice. An important recent study also tested in vitro the resistance of *M. lucifugus* fibroblast cell line to oxidative stress (96). Importantly *M. lucifugus* fibroblasts were also significantly more resistant to the effects of H_2O_2 then mouse cells (96). Bat cells also repair DNA damage faster then mouse fibroblasts (110). These results are consistent with the idea that evolution of long-lived bats required development of cellular resistance to oxidative stress. Interestingly, vascular cells (Figure 11) and fibroblasts (96) from bats do not exhibit superior resistance to paraquat (which elicits mitochondrial oxidative stress). These data provide

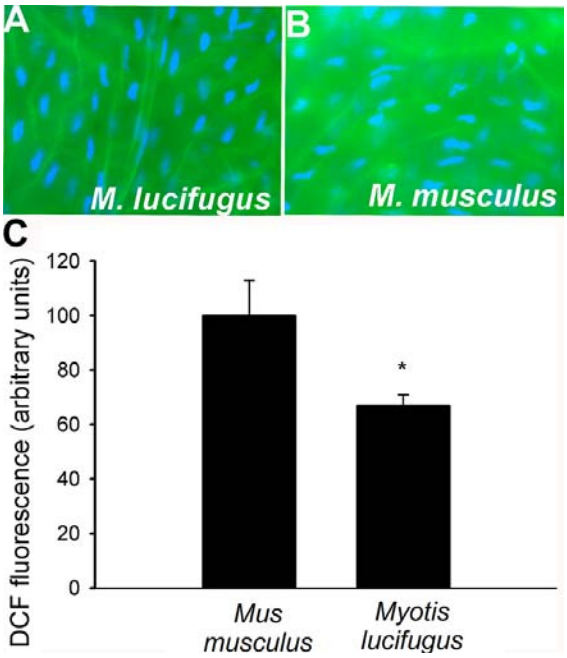

Figure 10. Decreased ROS production in long-lived *M. lucifugus*. Representative fluorescent images showing H_2O_2 production (measured by the C-H_2DCFDA fluorescence method (20, 92)) in endothelial cells of *en face* preparations of aortas of *M. lucifugus* (A) and mice (B). Green fluorescence: DCF, blue fluorescence: Hoechst-stained endothelial nuclei (original magnification: 10x). **C:** Bar graphs are summary data of DCF fluorescent intensities in endothelial cells of mice and *P. leucopus* (mean ± S.E.M. n=5 animals for each group).

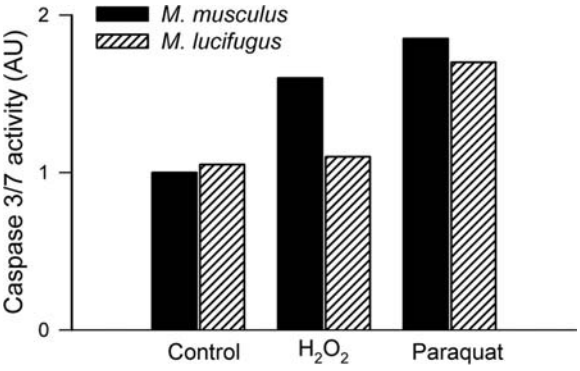

Figure 11. Oxidative stress resistance in long-lived *M. lucifugus*. Arteries of long-lived bats (*M. lucifugus)* tend to be more resistant to apoptotic stimuli than mouse vessels. Vessels were maintained in organoid culture and apoptotic cell death was induced by H_2O_2 (10^{-5} mol/L) or paraquat (3 mmol/L; which induced mitochondrial oxidative stress). Caspase 3/7 activity was assessed 10 h after apoptosis induction as described previously (20, 49, 83, 92).

justification for evaluation of cellular resistance to a much wider range of oxidative stressors in long-lived mammals.

6. PERSPECTIVES

Collectively, studies examining cellular and mitochondrial ROS production and oxidative stress resistance in long-lived species seem to concur in general with predictions based upon the oxidative stress theory of aging. On the basis of the aforementioned studies we predict that lower rate of cellular ROS generation will limit vascular damage and attenuate pro-inflammatory phenotypic changes contributing to the successful vascular aging of long-lived species.

Future studies should determine whether long-lived animals are more resistant to cardiovascular diseases, whose development is facilitated by oxidative stress (e.g. atherosclerosis, ischemia-reperfusion injury, vasculopathies associated with metabolic diseases). In humans diabetes mellitus is a major risk factor of cardiovascular disease. Thus, it will be interesting to see whether cells of longer-living animals are more protected against the adverse effects of high glucose. Also, it will be informative to compare resistance of cells of longer-living species to a much wider range of oxidative stressors as well. For example, previous studies showed that hepatocytes and fibroblasts from catalase overexpressing mice are more resistant to H_2O_2-induced cell death but are more sensitive to paraquat and TNFα toxicity (116). Because nitrosative stress seems to play an important role in cardiovascular pathophysiology and in the vascular aging process (8, 117, 118), it would be important to determine whether endothelial cells of long-lived species are also protected against the genotoxic action of reactive nitrogen species as well (8, 117). We would like to point out that although limiting apoptotic responses to oxidative stressors in blood vessels is likely beneficial, an increased rate of apoptosis may reduce the risk of cancers in parenchymal tissues. Because in mice cancer is the primary cause of death, further studies are needed to compare the pro-apoptotic effect of oxidative stress between long-lived species and mice in other cell types as well. The mechanisms underlying superior oxidative stress resistance in long-lived species are likely multifaceted. We are currently using cultured fibroblasts to investigate mechanisms related to ROS homeostasis and DNA repair that might be informative about successful aging of these species. It will be useful to establish primary endothelial cells from long-living species as well for studies into the mechanisms of vasculoprotection. One promising area of research is comparing redox sensitive pro-inflammatory signaling mechanisms in short-lived and long-lived animals. Will the same level of oxidative stress activate redox sensitive signaling pathways in a similar manner? Will they lead to similar endothelial activation and inflammatory gene expression? Tissues from aged *P. leucopus* and naked mole-rats are available, thus it will be possible to address whether age-related pro-inflammatory phenotypic changes are delayed in successfully aging species. Of special interest will be studies on age-related changes in TNFα, regulation of endothelial NAD (P)H oxidase, endothelial mitochondrial function and NF-κB signaling. We hope that this review will stimulate interest among vascular

biologists to consider long-lived species as study organisms in a comparative approach to cardiovascular aging research.

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Abbreviations: MLSP: maximum lifespan potential; LQ: longevity quotient; FAAH: fatty acid amide hydrolase; CAD: coronary artery disease; TNFα: Tumor Necrosis Factor-α

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Send correspondence to: Zoltan Ungvari, Department of Physiology, New York Medical College, Valhalla, New York 10595, USA, Tel: 914-594-3591, Fax: 914-594-4018, E-mail: zoltan_ungvari@nymc.edu

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