Published in final edited form as:
Mech Ageing Dev. 2010 ; 131(7-8): 527–535. doi:10.1016/j.mad.2010.06.002.

Mitochondrial oxidative stress and mammalian healthspan

Jonathan Wanagat[+], **Dao-Fu Dai**[††], and **Peter Rabinovitch**[††,*]
[††]Department Pathology, University of Washington, Seattle, WA 98195
[+]Department of Medicine, Division of Geriatrics, UCLA, Los Angeles, CA 90095

Abstract

Aging of the American society is leading to a growing need for disease-modifying interventions to treat age-related diseases and enhance healthspan. Mitochondria and mitochondrially-generated reactive oxygen species appear to play a central role in these processes and are a likely target for interventions. Conventional, untargeted antioxidants have not demonstrated a clear benefit in human studies. As a result, approaches have been developed to target antioxidants specifically to mitochondria. Studies have employed a wide array of targeted molecules including antioxidant enzymes such as catalase, peroxiredoxin, superoxide dismutases and small molecular compounds which recapitulate the antioxidant activities of these enzymes. Lifespan and healthspan effects differ between interventions suggesting varied roles for specific mitochondrial reactive oxygen species and their impact on usual aging. Consistent findings in myocardial protection across various interventions support a focus on the impact of cardiac aging on healthspan. The advancement of mitochondrially-targeted small molecule antioxidants suggests the prospect of swift translation to human use.

Keywords

Mitochondria; antioxidants; drug targeting; healthspan

Introduction

The aging of the American society, as predicted decades ago, is now upon us with an accelerating impact on society as a whole and specifically on healthcare delivery and expenditures. Diseases of aging have been largely refractory to standard approaches of modern medical research and for some of these, few if any, disease-modifying therapies exist (Butler et al., 2008). Additionally, numerous long-standing hypotheses of aging, often supported by an abundance of correlative data, lack clear evidence.

Mitochondria, mitochondrial function and mitochondrial ROS have been consistently implicated in many of these processes and disorders in a wide range of correlative studies (Wallace, 2005). However, the exact role of mitochondrial ROS remains unclear due to the correlative nature of the studies and the difficulty in modulating ROS *in vivo*. Despite these shortcomings, spending on vitamin supplements and conventional antioxidants reaches into

[*]Correspondence: Peter S. Rabinovitch, MD, PhD., Professor, Department of Pathology, University of Washington, 1959 Pacific Ave NE, HSB-K081, Seattle, WA 98195, Phone: 206-685-3761; Fax: 206-616-8271, petersr@u.washington.edu.

the tens of billions of dollars annually, without strong evidence of positive effects and some evidence suggestive of harm (Lippman et al., 2009).

Early approaches, in the field of biogerontology, to test the role of mitochondrial antioxidants focused on disrupting various antioxidant enzymes, generating abundant literature with generally negative results for aging phenotypes (Perez et al., 2009). While this contradicts the interpretations of earlier paradigms, this approach does not address the significance of increased resistance to mitochondrial ROS.

The need for models to test causal relationships led to the generation of genetic and small molecule models of mitochondrially-targeted antioxidants. We first review the genetic models of mitochondrially-targeted antioxidants including mitochondrial catalase (MCAT), peroxiredoxin 3 (Prdx 3), mitochondrial glutathione peroxidase 4 (mGPX4) and manganese-superoxide dismutase (SOD2). We then examine studies of an emerging class of mitochondrially-targeted small molecule antioxidants including mitoQ, mitoplastoquinones and the Szeto-Schiller (SS) peptides.

Genetic models of mitochondrially targeted antioxidants

A number of mouse models have been genetically engineered to facilitate more direct hypothesis testing by studying the effects of increased mitochondrial antioxidant potential. These mouse models involve overexpression of endogenous mitochondrial antioxidants including: peroxiredoxin 3 (PRDX3), mitochondrial glutathione peroxidase (mGPX), and manganese superoxide dismutase (MnSOD, SOD2) and retargeting of a peroxisomal antioxidant to mitochondria in the case of the mitochondrial catalase (MCAT) mouse. These models have been amenable to the study of usual aging and age-associated diseases as well as other disorders in which mitochondrial oxidative stressors are implicated. In the following sections, we review these models and the insight they have provided into the role of mitochondrial ROS and oxidative damage in aging.

Mitochondrial catalase (MCAT)

The MCAT mouse model was one of the first genetic models of a mitochondrially-targeted antioxidant and the only example of an exogenous enzyme that is not normally found in mitochondria. We will review the initial report from Schriner et al. (2005) showing life span extension, protection from mitochondrial ROS and oxidative damage and some early indicators of healthspan effects. This is followed by our end of life (EOL) analyses of cancer and other causes of death and studies of protective MCAT effects on the cardriomyopathy of aging. We then review the growing number of studies using the MCAT model to test the role of mitochondrial H_2O_2 in usual aging and other age-related diseases including presbycusis, AZT-induced cardiomyopathy, insulin sensitivity, exercise performance, and retinal cell loss in retinitis pigmentosa.

MCAT effects on oxidative damage, age-related pathology and lifespan

In 2005, Schriner et al., reported their work to delineate the role of H_2O_2 and oxidative damage in aging and age-related diseases (Schriner et al., 2005). They developed six founder lines of transgenic mice expressing three types of human catalase: 1) peroxisome-targeted catalase (PCAT); 2) nuclear-targeted catalase (NCAT); and 3) mitochondrial-targeted catalase (MCAT). MCAT expression was highest in brain, heart and skeletal muscle with levels of catalase activity in the heart of MCAT mice approximately 50-fold higher than WT littermates.

PCAT extended median lifespan by ~10% without significant extension of maximum lifespan in two founder lines, while NCAT did not have a significant lifespan effect in mice. In contrast, MCAT extended both mean and maximum lifespan by 4.5-5.5 months (17-21%) and 4.5

months, respectively, in the two founder lines. Lifespan extension in the MCAT mice was not sex specific and none of the six founder lines displayed differences in body weight or food consumption as compared to WT littermates.

In the initial report, differences in cancer and cardiac phenotypes were noted in the MCAT mice as compared to WT littermates and these will be discussed in more detail in later sections. The MCAT effect on free radicals and oxidative damage was shown in assays of H_2O_2 production, aconitase activity, 8OHdG levels and mtDNA deletion products. Hydrogen peroxide generation in cardiac mitochondria was ~33% lower in MCAT mice while aconitase activity was protected after treatment of mitochondria with exogenous H_2O_2. 8OHdG levels were significantly lower in MCAT skeletal muscle from 26-29mo mice, but were not different in cardiac tissues. MtDNA mutation load has been used widely as a readout for oxidative mitochondrial damage. Whole mitochondrial genome PCR from total DNA isolates of heart and skeletal muscle showed fewer deletion products in MCAT skeletal muscle at 18-22 months, but no significant MCAT effect in heart at those ages or in either tissue at 33 months. More sensitive mtDNA mutation assays have since been performed on purified mtDNA and show a lower mutation load in MCAT mice, as discussed in a later section.

Initial findings of MCAT protection from cancer and the cardiomyopathy of aging led to more detailed studies in these areas which have been recently published and are discussed in the following sections.

MCAT effects on end of life pathologies and cancer

Cancer is a major determinant of mammalian lifespan, particularly in rodent models. Mice in the original MCAT cohort were noted to have a trend toward reduced incidence of splenomegaly and splenic lymphoid neoplasia which are common findings in the WT C57BL/6J genetic background. This cursory look at cancer phenotypes did not reach statistical significance but prompted a concerted search for MCAT effects on cancer phenotypes and a broader analysis of MCAT effects on mouse end of life (EOL) pathologies.

Treuting et al. (2008) set out to examine more closely the impact of MCAT expression on murine cancer phenotypes and EOL pathology. Studies were conducted on the two original MCAT founder lines that had been backcrossed for greater than 10 generations. These mice were raised as an aging colony under specific pathogen free conditions. Mice used for histopathological analyses were considered to be at EOL if moribund as demonstrated by one or more clinical signs suggestive of imminent death in the succeeding 24 hours. EOL pathologies were then determined through standardized necropsies of each animal and neoplastic processes, separated into hematopoetic and non-hematopoietic classes, were graded according to Ikeno et al. (2003). Grading the severity of neoplastic and non-neoplastic lesions facilitated estimations of disease burden and cause-of death-assignment criteria (Kodell et al., 1995) allowed assessment of contributing causes of moribundity.

As hypothesized, based on the initial study, MCAT expression was associated with reduced nonhematopoietic tumor burden from 26% of WT mice to 17% of MCAT mice. Most significantly, the burden of *malignant* nonhematopoietic tumors was reduced 8-fold in mCAT mice (p=.007). Nonhematopoietic tumors were predominantly adenocarcinomas and adenomas. Cardiac lesions were also significantly reduced. Lastly, there was a statistically insignificant trend towards reduced systemic inflammation observed as amyloid deposition in various organs including intestines and renal glomeruli. The protection from nonhematopoietic tumors, cardiac lesions and trend toward protection from systemic inflammation occurred without effects on the prevalence or severity of hematopoietic neoplasia or glomerulonephropathy commonly observed in this mouse strain. Combined disease burden and comorbidity at EOL were also reduced. Importantly, as mentioned in the original report

(Schriner et al., 2005), MCAT expression is not associated with any known detrimental effects on reproduction, development, pathology or behavior as are commonly found in other murine models of life-span extension.

The results suggest that oxidative damage is involved in aging of C57BL/6J mice via modulation of a subset of age-associated lesions. Cancer effects suggest the ability of MCAT expression to modulate cancer initiation, progression or metastasis. This study again highlighted the importance of MCAT expression in protecting from the cardiomyopathy of aging as discussed in the following section.

Cardiac effects of MCAT

Observations from the first two MCAT studies suggested important MCAT effects on cardiac aging. This was not unanticipated as the heart possesses robust MCAT transgene expression in combination with the very high cardiac mitochondrial content and complete reliance on mitochondrial energy production. Moreover, the mitochondrial dependence occurs in the setting of a post-mitotic tissue largely incapable of replacing damaged or lost cardiomyocytes. The next two sections review the protective effects of MCAT from the cardiomyopathy of aging and from the mitochondrial toxicity of zidovudine (AZT, azidothymidine).

Effects of MCAT on the cardiomyopathy of aging

Cardiovascular diseases dominate the common contributors to mortality in the US and age is the primary risk factor (NCEP ATP III, Expert Panel on Detection, 2001). ROS and mitochondrial damage have long been implicated in the cardiomyopathy of aging, but direct evidence has been lacking. The studies of Schriner et al., (2005) and Treuting et al., (2008) suggested substantial cardiac sparing effects of MCAT. To build on those initial observations, we used echocardiography to measure cardiac function in aging WT and MCAT mice ranging in age from 4 to greater than 24 months of age. In addition to echocardiographic parameters of myocardial structure and function, we assessed mitochondrial oxidative damage and mtDNA mutations and cellular pathways implicated in cardiac hypertrophy including the ERK, calcineurin-NFAT and angiotensin II signaling pathways.

Echocardiography in C57BL6/J mice showed that aging mouse hearts recapitulate the usual pattern of human cardiac aging despite the lack, in these mice, of the well-characterized, modifiable human cardiac risk factors (e.g., dyslipidemia, diabetes, and HTN). These age-related changes included hypertrophy and worsening of systolic and diastolic cardiac function. *In situ* histological analyses of dissected hearts validated the echocardiographic measurements by showing increased ventricular wall thickness, cardiomyocte hypertrophy, and proliferation of myocardial fibrosis. All of the functional and structural age-related changes in the mouse heart were significantly reduced by MCAT expression.

Mitochondrial changes in the aging mouse heart included an ~30% increase in protein carbonyls and mtDNA copy number with an ~3-fold increase in mtDNA point and deletion mutations. The changes in mtDNA copy number suggested the involvement of mitochondrial biogenesis pathways and analyses showed age related increases in PGC-1alpha, TFAM, NRF-1 and NRF-2. All of the age-related mitochondrial changes in the mouse heart were ameliorated by MCAT with the exception of biogenesis signaling through NRF-1 and -2 which showed a trend toward an MCAT effect that was not statistically significant.

A number of cellular pathways are involved in cardiac hypertrophy including phospho-ERK1/2 and calcineurin-NFAT (nuclear factor of activated T-cells). We examined both of these in aging mouse hearts and found no alteration of phosphorylated or total ERK1/2 levels. The calcineurin-NFAT pathway, however, was activated with aging as were downstream cofactors

such as GATA4 and NFAT target genes including MCIP-1 (modulatory calcineurin interactin protein-1) and ANP/BNP (atrial and brain natriuretic peptides). Once again, aging changes in these pathways were attenuated by MCAT expression.

Diastolic dysfunction is very common among older individuals, particularly older women with a history of hypertension, and there are few effective therapies. Changes in myocardial compliance and calcium handling are thought to be involved and MCAT maintains compliance through decreases in hypertrophy and fibrosis as mentioned earlier. We also demonstrated that age-related changes in calcium handling were preserved in MCAT mice, likely through preservation of SERCA2 (sarco[endo]plasmic reticulum Ca ATP-ase) activity.

The blockade of angiotension receptors has been shown to prevent many of the age-related changes in the mammalian heart including those we observed in C57BL/6J mice. With the broad effects of MCAT expression on these changes, we examined the steady state tissue levels of angiotension II and found that these increased with age in the mouse heart. Interestingly, MCAT did not reduce angiotensin II levels, a finding which suggests that MCAT expression works distal to the angiotension receptor in this signaling pathway.

With the striking changes in cardiac physiology with aging, we examined univariate Cox regression analyses using age, myocardial performance index (MPI) and left ventricular mass index (LVMI) as predictors. All of these were significant survival predictors and a multivariate Cox model was used to generate a cardiac aging risk score. This cardiac aging risk score provided a clinically relevant measure of mortality risk with mice in the highest tertile showing a significantly increased risk of all-cause mortality with a hazard ratio of 2.88 (95% confidence interval, 1.43-5.82, p=0.003). MCAT expression shifted the distribution of cardiac aging risk scores to lower tertiles as compared to WT mice. Moreover, the attributable risk function in the proportional hazards model suggested that 55% of the mortality risk was attributable to the increases in MPI and LVMI.

Cardiac studies in the MCAT mouse aging model demonstrate that, just as in human aging, cardiac disease plays a significant role in determining life- and health-span. The studies demonstrate the power of echocardiography as a non-invasive technique which provides longitudinal analyses of cardiac structure and function. In C57BL/6J mice, this appears to be a useful predictor of life-span. Future studies using the MCAT mouse may help to delineate the role of mitochondrial ROS in other cardiac diseases such as heart failure, ischemia-reperfusion and hypertrophy.

MCAT protection from AZT-induced cardiomyopathy

Harman's original free radical hypothesis implicated both endogenous and exogenous free radicals in the pathophysiology of aging. Since that time, numerous exogenous oxidants and compounds which promote mitochondrial ROS production have been identified. Some of these compounds have therapeutic uses that are accompanied by off-target effects that promote ROS production or mitochondrial dysfunction. Zidovudine (3′-azidothymidine, AZT) is an example of such a compound. AZT is an important nucleoside reverse transcriptase inhibitor with dose limiting toxicities related to mitochondrial toxicities (Lamperth et al., 1991). As the HIV population rapidly ages, we are only beginning to learn about the interface between aging, HIV infection and HIV treatment regimens, but early data suggest that they will negatively synergize (Effros et al., 2008).

Kohler et al. (2009), examined the effect of MnSOD overexpression and MCAT on AZT-associated cardiomyopathy in the mouse model. AZT was given by daily gavage at a dose of 0.22mg per day, a dose which the authors state reflects clinically relevant human doses. AZT treatment for 35 days in wild-type mice increased LV mass and LVEDD (as measured by

echocardiography), heart mitochondrial H_2O_2 production (as measured by Amplex Red Assay from Invitrogen) and cardiomyocyte damage while disrupting aconitase activity and mitochondrial structure (demonstrated by electronmicrography). MnSOD overexpression and MCAT prevented the AZT-induced damage and preserved myocardial structure such that mice with these transgenes did not differ from vehicle-treated control mice. Interestingly, MnSOD overexpression did not affect heart mitochondrial H_2O_2 production, but MCAT lowered production by ~50%. Conversely, MnSOD heterozygous knockout mice suffered greater damage with AZT treatment leading to 1.5-fold increases in LV mass and heart mitochondrial H2O2 production and ~33% decrease in aconitase activity. These findings point to the role of H_2O_2 in AZT-induced cardiomyopathy and the therapeutic potential of mitochondrially-targeted antioxidants.

Preservation of glycemic control by MCAT expression in fat fed mice

Changes in body composition and insulin sensitivity are hallmarks of usual mammalian aging and mounting evidence suggests these processes are linked through mitochondrial function and cell signaling through reactive oxygen species and cell redox balance (Evans et al., 2002). This was elegantly demonstrated using two mitochondrially targeted H_2O_2 scavengers – the MCAT mouse model and SS-31 peptide, a mitochondrially targeted antioxidant peptide that is discussed in detail later in this review (Anderson et al., 2009). Anderson et al. placed rodents on one of three diet regimens differing in their fat content: 1) standard chow (generally ~15% fat), 2) 100% fat (lard) for 72 hours or 60% fat for 3 weeks. After the prescribed time on the diets, the propensity for mitochondrial H_2O_2 production in permeabilized muscle fibers was tested by succinate titration during state 4 respiration. Mice or rats on the acute or sub-acute high fat diets showed a 3- to 4-fold increase in maximal H_2O_2 production. The increase in H_2O_2 production was associated with increases in GSSG and reduction in the GSH/GSSG ratio indicating a fat-induced shift to an oxidative cellular environment and insulin resistance as measured by hyperinsulinemic-euglycemic clamp. MCAT mice on the high fat diet had much lower rates of muscle fiber H_2O_2 production, preserved muscle GSH levels and maintained normal insulin sensitivity. These findings were recapitulated using the SS-31 peptide.

MCAT effects on exercise performance

A dual role for reactive oxygen species in skeletal muscle has long been postulated, as a precursor to damage and required for normal muscle function. To outline the role of H_2O_2 in these processes, Li et al. (2009) developed an AAV-9 MCAT vector that was used to infect neonatal C57Bl/6 mice resulting in systemic MCAT expression. MCAT expression was confirmed in heart and skeletal muscle in infected mice after three months. The MCAT expression in these mice was 10-fold higher than endogenous catalase activity in the heart, and 3 to 5-fold higher in other muscles except for soleus where catalase activity was not significantly higher than in uninfected control mice. Immunohistochemistry demonstrated co-localization of AAV-MCAT with cytrochrome *c* oxidase (complex IV), verifying mitochondrial targeting. In this MCAT model, the infected mice demonstrated enhanced treadmill performance as measured by exhaustion limited running distance. Interestingly, the AAV-MCAT did not change the specific or absolute force of isolated EDL muscles. Exercise endurance could thus be secondary to increased cardiac function and improved muscle perfusion given our findings of improved cardiac physiology in MCAT mice (Dai et al., 2009). As with other MCAT transgenic mouse models, the AAV-9 MCAT mouse did not demonstrate negative effects on muscle mass or body weight. The challenge in studies of muscle physiology and mitochondrial function is to identify physiological assays which are able to detect mitochondrial functional changes with high sensitivity rather than focusing on function of the muscle fiber contractile machinery.

MCAT protection from blindness in a model of retinitis pigmentosa

Prevention of oxidative damage by alteration of endogenous antioxidants will require consideration of the tissue, the mechanism of ROS generation and secondary effects of antioxidant modulation (e.g., production of other ROS or changes in redox signaling pathways). Such a scenario is exemplified in a recent study of antioxidant defenses in retinitis pigmentosa (RP). RP is a family of photoreceptor diseases characterized by a pathognomonic clinical progression from night blindness to tunnel vision and eventual blindness. Dozens of genetic alterations have been linked to RP and include autosomal dominant, recessive or X-linked patterns of inheritance. The blindness in RP results indirectly from the death of cone cells which succumb to oxidative damage resulting from the loss of rod cells and the subsequent dysregulated increase in retinal tissue oxygen levels. This explains how diverse mutations causing rod cell death have convergent effects on cone cells. Usui et al. (2009) tested whether increases in endogenous enzymatic antioxidants could prevent cone cell death in retinitis pigmentosa. To accomplish this, they increased endogenous enzymatic antioxidant defenses – SOD1, SOD2 and MCAT. Transgenic mice were generated for SOD1 and for SOD2 and MCAT, inducible expression in rods and cones by driving expression with a reverse tetracycline transactivator/interphotoreceptor retinol-binding protein promoter. These overexpressing mice were crossed with mouse models of retinitis pigmentosa ($rd1^{+/+}$ and $rd10^{+/+}$). Levels of oxidative damage were assessed by protein carbonyl, hydroethidine staining for superoxide, histological measurement of cone cell density and cone cell function by scotopic electroretinograms (ERGs) on intact mice.

Overexpression of SOD1 in $rd1^{+/+}$ mice led to ~25% increase in protein carbonyls and reduced cone cell density and function as compared to $rd1^{+/+}$ mice with normal SOD1 expression. Thus, cytoplasmic expression of an antioxidant enzyme, in this care, demonstrates a paradoxical increase in oxidative damage and cell death. Tetracycline-induced overexpression of SOD2 or MCAT alone did not affect retinal carbonyl content, cone cell survival or cone cell function by scotopic ERGs. However, simultaneous tetracycline-induced overexpression of SOD2 and MCAT in $rd10^{+/+}$ mice reduced *in vivo* retinal superoxide levels to those found in WT control mice and decreased retinal protein carbonyl content by ~25% as compared to $rd10^{+/+}$ mice. Concomitant with decreased superoxide and protein carbonyls, mice with combined SOD2 and MCAT overexpression demonstrated preserved cone cell density and function.

Thus, overexpression of a variety of cellular antioxidant enzymes shows dependence of efficacy on type of antioxidant activity, timing of expression and subcellular localization.

MCAT amelioration of presbycusis

Presbycusis, or age-related hearing loss, is a common and well-defined clinical aspect of usual mammalian aging. Nearly half of Americans over 65 suffer from presbycusis with obvious impacts on socialization, isolation and independent living. Prolla and colleagues examined the roles of apoptosis and oxidative damage in presbycusis. Mice with a C57BL/6J background were the experimental model. As with many laboratory mouse strains, these mice develop presbycusis, which occurs by 12 to 15 months of age in this strain. To test role of apoptosis, they used two KO mouse models, $Bak^{-/-}$ and $Bax^{-/-}$, genes which express proapoptotic proteins important in apoptosis triggered through the mitochondrial pathway. Role of oxidative stress in presbycusis, tested using administration of paraquat as an induction of mitochondrial oxidative stress and the MCAT mouse and mice fed a variety of orally available antioxidants as models of decreased oxidative stress.

Knockout of Bax or Bak genes had disparate effects on cochlear apoptosis and presbycusis. Modulation of apoptosis by elimination of Bak activity largely prevented presbycusis and cochlear apoptosis, but loss of Bax activity had no significant effect. Having found that

apoptosis in prebycusis was mediated by Bak, they examined the role of oxidative stress by administrating paraquat to WT and $Bak^{-/-}$ mice. Lack of Bak activity protected mice from paraquat-induced cochlear apoptosis which was associated with a 3-fold increase in Bak transcript expression in WT mice. The effect of paraquat on presbycusis and cochlear Bak levels implicated oxidative stress and this was tested further through use of the MCAT mouse. At 13 months of age, MCAT mice displayed significant protection from cochlear cell loss and presbycusis. MCAT protection from presbycusis was associated with reduced 8-oxodG and lower Bak expression.

The success of a transgenic antioxidant led Prolla's team to examine the role of orally available antioxidants in protection from oxidative stress induced, Bak-mediated cochlear damage. Of 17 antioxidant compounds tested, three compounds (alpha-lipoic acid, coenzyme Q10, and N-acetylcysteine) demonstrated a protective effect on hearing, prevented apoptotic cell loss and lowered Bak expression. The authors point out that non-selective antioxidants which are not targeted to mitochondria did not have the same efficacy in protecting against presbycusis. As only one age group was studied, additional studies may help elucidate the durability of protection and required timing of therapy.

MCAT conclusions

In summation, the MCAT mouse studies suggest that mitochondrial ROS and H_2O_2 in particular are causally involved in moderating mammalian life- and healthspan. Importantly, MCAT expression in these many studies has not demonstrated negative effects on usual mouse development or physiology. A new genetically-engineered MCAT models has been generated to facilitate the spatial and temporal control of MCAT expression to help better pinpoint its protective effects. This mouse strain was engineered with MCAT inserted into a human GAPDH BAC to maximize uniformity of expression and minimize positional effects. A floxed stopper sequence prevents MCAT expression until Cre-mediated recombination occurs. In addition to more uniform expression, the BAC model shows more ubiquitous tissue distribution (Wanagat, unpublished observations). This mouse can be crossed with a variety of Cre-expressing mice to control the timing and localization of MCAT expression.

Transgenic mouse models of endogenous, mitochondrially-targeted antioxidant enzymes

Several transgenic mouse models of mitochondrially-targeted antioxidants utilize the overexpression of endogenous antioxidant enzymes to target the metabolism of specific ROS molecules (e.g., SOD2 overexpression to metabolize superoxide) or protect specific cellular components (e.g., mitochondrial glutathione peroxidase 4 protection from lipids peroxidation). We review three of these models including: 1) peroxiredoxin 3, 2) mitochondrial glutathione peroxidase 4 and 3) manganese-superoxide dismutase. These models generally show the intended protection from ROS, but differ in their health- and lifespan effects – even between studies of similar transgenic mouse models.

Peroxiredoxin 3 overexpression protects against exogenous oxidative stress and a high fat diet

Peroxiredoxin 3 (Prdx3) is an endogenous antioxidant that is normally located exclusively in the mitochondrial matrix. Recently Chen et al. (2008), generated a mouse with transgenic Prdx3 (TgPrdx3) overexpression. Levels of 3- to 5-fold Prdx3 overexpression were attained in a variety of tissues including brain, heart, liver, kidney and skeletal muscle. H_2O_2 production was lower in isolated brain and skeletal muscle mitochondria from TgPrdx3 mice without changes in ATP generation or membrane potential. This reduction in H_2O_2 production correlated with decreases in DCF fluorescence, F_2-isoprostanes and mitochondrial 4-hydroxynonenal in the TgPrdx3 mice. Resistance to exogenous oxidative stressors was increased in isolated TgPrdx3 fibroblasts including resistance to paraquat, H_2O_2 and cadmium.

Strikingly, even young TgPrdx3 mice up to 6 months of age displayed alterations in glucose metabolism. TgPrdx3 mice have reduced fasting blood glucose levels and increased glucose tolerance with a trend toward lower serum insulin. TgPrdx3 mice on a high fat diet (58% fat from lard, 25.6% carbohydrate and 16.4% protein) have similar changes in glucose and insulin regulation. Chen et al. further demonstrated that TgPrdx3 overexpression may be affecting these metabolic changes through PI3K/Akt signaling and increased GSK3 phosphorylation. Studies examining the efficacy of Prdx3 in protection during other physiologic stressors or disease state may be quite revealing.

Glutathione peroxidase provides in vivo protection from exogenous oxidative stress

Glutathione peroxidase 4 is another endogenous antioxidant defense enzyme that is targeted to mitochondria (mGPX4) as well as other cellular compartments. The unique activity of mGPX4 in the metabolism of lipid and lipoprotein hydroperoxides suggests its role in protection of mitochondrial membrane lipids from ROS damage (Ursini and Bindoli, 1987). The role of mGPX4 in protecting mitochondrial function from oxidative damage was tested directly by Liang et al. (2007), when they generated a transgenic mouse with systemic overexpression of GPX4 (TgGPX4). Liver mitochondria from WT or Tg(GPX4) mice showed no difference in ATP production rate, however, pre-treatment with diquat reduced ATP production in WT mice, but did not affect ATP production in Tg(mGPX4) mouse mitochondria. The protection of ATP production by Tg(mGPX4) was not due to preservation of ETC activities as these were reduced in diquat treated animals, regardless of genotype. However, mitochondrial membrane potential was spared in the Tg(mGPX4) mice following diquat treatement, suggesting maintenance of membrane potential by reduction of proton leakage as a likely mechanism.

Mixed effects of MNSOD overexpression on oxidative stress resistance and lifespan

The overexpression of manganese superoxide dismutase (MnSOD or SOD2) lowers superoxide production, but has conflicting effects on mouse lifespan (Ho et al., 1998; Hu et al., 2007; Jang et al., 2009). In the first mouse model, SOD2 overexpression was obtained by generating transgenic B6C3 mice with the human beta-actin promoter driving human SOD2 gene expression, resulting in wide tissue expression including a 5-fold increase in lung tissue (Ho et al., 1998). SOD2 overexpression in these mice offered some protection against respiration in an atmosphere of 90% oxygen, but no protection from >99% oxygen exposure.

In 2007, Hu et al., used the same transgenic SOD2 overexpressing mouse line to examine cognitive function and lifespan. In the brain, SOD2 overexpression in this model ranged from 2.6-fold in the midbrain to 4.6-fold in the striatum. They found that SOD2 overexpression did not alter the usual cognitive decline in synaptic plasticity or memory in two year old transgenic mice as compared to WT controls. The lack of protection from cognitive decline occurred despite a decrease in mitochondrial superoxide generation in hippocampal neurons. Hu et al., (2007) also examined the lifespan effect of SOD2 overexpression in a small cohort comparing 30 WT mice to 24 transgenic mice. Mean lifespan was increased 4% from 27.6 to 28.8 months, while maximum survival of the longest lived mouse differed by 18% with the TgSOD2 mice living to 43 months compared to 36.5 months for the longest lived WT mouse. Of the original 24 TgSOD2 mice, four mice (18%) survived greater than 40 months.

In contrast, recent work by Jang et al., (2009) using a different line of SOD2 overexpressing mice did not demonstrate a life-extension effect. In this study, SOD2 expression was from the native promoter of the mouse SOD2 gene. These mice had SOD2 activity approximately 2-fold higher than controls, again in a wide range of tissues, and this expression persisted to at least 26-28 months of age. This level of SOD2 overexpression prevented lipid peroxidation, increased resistance to paraquat treatment and lessened the usual age-related drop in skeletal

muscle mitochondrial ATP production. However, longitudinal study of 47 WT and 50 transgenic mice showed no lifespan effect with this level of SOD2 overexpression.

The SOD2 studies highlight many of the potential complicating factors regarding mitochondrial targeting of antioxidant enzymes, including threshold effects, targeting of specific ROS, and background effects of genetically-engineered models. Threshold effects are common in mitochondrial biology and, particularly, in age-related mitochondrial dysfunction (Herbst et al., 2007; Perez et al., 2009). Therefore, the differences in SOD2 expression in these two models and in other models is not easily dismissible and titration of gene expression may be critical in demonstrating protective effects. The original free radical hypothesis of aging broadly implicated all ROS and all oxidative damage in aging and disease. The above studies clearly establish that modulation of specific ROS and specific oxidative damage will have varying downstream effects. The increasing numbers of these models targeting different ROS components and pathways will help focus the hypothesis. Lastly, as in all mouse models, the genetic background and species specific pathologies (e.g., cancer in the mouse models) cannot be overlooked as potential confounders. In the next section, we review small molecule antioxidants which may help circumvent some of these limitations while simultaneously moving us toward therapeutic interventions applicable to human use.

Small molecule mitochondrially targeted antioxidants

The successes of the enzymatic mitochondrially-targeted antioxidants discussed above point to the need for pharmaceutically viable, small-molecule antioxidants. However, most small-molecule antioxidants distribute throughout the body, tissue and cellular compartments, with only a small fraction being taken up by mitochondria. In this review, we focus on currently available mitochondrially-targeted antioxidant small molecule compounds which are primarily H_2O_2 scavengers and have demonstrated efficacy in cell culture and animal models and data which suggest that they will extend health span. We will first review the work of Michael Murphy and colleagues and their class of lipophilic cations (e.g., mitoQ), then briefly review similar work using plastoquinone derivatives, and lastly examine mitochondrially-targeted peptide compounds developed by Hazel Szeto and Peter Schiller. Targeting of antioxidant compounds to mitochondria has, thus far, followed two main approaches that differ in the chemical group responsible for targeting and the dependence on active mitochondrial membrane potential.

MitoQ (mitoquinone)

Murphy and coworkers used an approach that utilized conjugation of a triphenylalkylphosphonium (TPP) cation to a variety of antioxidants such as coenzyme Q, vitamin E, and ebselen (Cocheme et al., 2007; Filipovska et al., 2005). This approach has the benefit of being orally bioavailabe, but requires functional mitochondria as uptake of the TPP cation is dependent on mitochondrial membrane potential (Kelso et al., 2001).

$MitoQ_{10}$ is comprised of the ubiquinol moiety of coenzyme Q conjugated to the TPP cation by a ten carbon chain. MitoQ compounds are taken up rapidly by mitochondria and adsorb to the matrix surface of the inner membrane where it is reduced to the active ubiquinol antioxidant by electron transport complex II (Asin-Cayuela et al., 2004; Kelso et al., 2001). The length of the aliphatic side chain appears to determine membrane partitioning, with longer side chains binding with greater affinity to lipid bilayer than short chains (Asin-Cayuela et al., 2004). Early reports showed the ability of $MitoQ_{10}$ to decrease H_2O_2 efflux from mitochondria as determined by decreased dichlorofluorescein fluorescence (Murphy and Smith, 2007). The exact mechanism is unclear, as $MitoQ_{10}$ does not directly act with H_2O_2, but could be acting upstream of H_2O_2. It does not decrease superoxide radicals but does decrease lipid peroxidation. Subsequent tissue culture studies showed that mitoQ protects cells from oxidative

damage, telomere shortening and lengthens replicative lifespan (Jauslin et al., 2003; Saretzki et al., 2003). In a study of TNF induced necrosis of L929 cells, mitoQ was not as effective as the antioxidant BHA (butylated hydroxytoluene antioxidant), but it was shown that BHA may work through changes in mitochondrial coupling rather than directly on mitochondrial ROS generation (Jarvis et al., 2007).

The effects of mitoQ on lifespan were first studied in the model organism *Drosophila melanogaster* by Magwere et al., (2006). In these experiments, a number of antioxidant compounds including mitoQ were tested for their ability to protect flies from increased oxidative stress and for effects on fly life-span. The models on increased oxidative stress included flies lacking SOD1, flies with decreased expression of SOD2 or flies exposed to paraquat, while lifespan studies were conducted on WT flies. MitoQ protected against these various oxidative stresses but did so in a manner dependent on sex and developmental stage. The same held true for the lifespan effects of mitoQ, where female flies showed dose dependent decreases in lifespan whether fed as larvae or adults. In contrast, male flies showed no lifespan effects when fed mitoQ as larva and a shortened lifespan with feeding as adults. The authors reviewed a number of limitations to their use of mitoQ including the lack of information pertaining to the pharmacokinetics and pharmacodyamics of mitoQ in flies.

There are a growing number of reports of the effects of mitoQ on mammalian health with many of these focusing on cardiac physiology. To test the efficacy of mitoQ in a rodent cardiac ischemic-reperfusion model, Adlam et al. (2005) orally administered mitoQ and control compounds to rats for 14 days before Langendorf preparation of excised hearts. Ventricular function was measured before and after 30 minutes of ischemia and 60 minutes of reperfusion. MitoQ significantly protected the young rat heart from functional, structural and cell death changes following ischemia reperfusion. Functional changes protected by mitoQ included increased left ventricular developed pressure, increased maximal rate of pressure change, coronary flow and heart rate as compared to control fed rats. Pre-treatment with mitoQ significantly decreased cell death as evidence by decreased LDH levels in the cardiac perfusate and prevented mitochondrial structural changes. Protective effects of mitoQ on cardiac function and structure may be mediated by protection of mitochondrial function. RCR, state 3 respiration and complex I activity were all significantly and dramatically protected from ischemia by mitoQ in this model.

The *ex vivo* findings would be strengthened by *in vivo* studies and two recent studies of mitoQ and cardiac function have addressed this. A sepsis model in the rat recapitulated the protective mitoQ effects in the ex vivo hanging heart model (Supinski et al., 2009). In this study, sepsis-induced cardiac dysfunction was replicated by intraperitoneal injection of endotoxin and mitoQ was delivered orally in the drinking water. As in the ischemia-reperfusion study, mitoQ prevented endotoxin induced declines in RCR, state 3 respiration and ATP production and concomitantly protected *ex vivo* cardiac function. In the spontaneously hypertensive rat, eight weeks of oral mitoQ therapy reduced systolic blood pressure by 25mmHg, protected from the cardiac hypertrophic response and improved endothelial function (Graham et al., 2009).

The therapeutic potential of mitoQ is suggested in recent studies investigating the long term effects of mitoQ in mice and early human studies. Treatment of 4-6 week old C57BL/6 with up to 6.5 months of mitoQ via drinking water showed no negative physiological effects and no evidence for a pro-oxidant activity at the test doses (Rodriguez-Cuenca et al., 2009). Interestingly, body fat stores were lower in the treated mice and were reflected in decreased serum triglyceride levels, without changes in cholesterol or free fatty acids.

Two human trials have been referenced in recent publications (Gane et al., 2008; Snow et al., 2008). Both were Phase II trials to examine efficacy in small populations. Treatment of

Parkinson's disease patients for up to one year showed no adverse effects, but also no evidence of disease modification (Snow et al., 2008). The second trial, in patients with chronic hepatitis C, examined randomized treatment with two doses of mitoquinone (40mg or 80mg) (Gane et al., 2008). The primary endpoints after 28 days of therapy were serum alanine aminotransferase levels (alanine aminotransferase, ALT). The abstract reports that serum ALT levels were improved from baseline in the mitoquinone-treated subjects ($p<0.05$), but compared to placebo control, the ALT decline was not statistically significant with an ALT decline of 45 ± 10.4 U/l in treated patients versus 9 ± 14.3 U/l in untreated patients ($p=0.054$). A number of details cannot be gleaned from the abstract including dose effects, method of randomization and blinding, or durability of treatment.

Mitochondrially targeted plastoquinones

Use of the plastoquinone moiety found in plant chloroplasts for mitochondrial targeting is hypothesized to capitalize on the unique characteristics of these molecules. An extensive review of these compounds was recently published, so we will not attempt to recapitulate that in this article (Skulachev et al., 2009) Studies with one of the compounds, (10-[6′-plastoquinonyl] decyltriphenylphosphonium, SkQ1), demonstrated dramatic increases in lifespan across species including *Podospora anserine, Ceriodaphnia affinis, Drosophila melanogaster* and outbred SHR mice, that are not related to the inbred SHR rat strain. The median lifespan in SHR mice increased from 388 days in untreated mice to 512-522 days in treated mice on doses of SkQ1 ranging from 0.5 to 50nmol/kg/day. A histopathological evaluation of the aging SkQ1-treated or control SHR mice was not presented but may provide some insight into the protective mechanisms of this compound. Other reports in the same journal issue demonstrated the ability of SkQ1 to prevent a wide range of cardiac, renal and ocular diseases as well as tumor development in mice (Agapova et al., 2008; Anisimov et al., 2008; Antonenko et al., 2008; Bakeeva et al., 2008; Neroev et al., 2008). These results offer further support of the utility of mitochondrially targeted antioxidants as therapeutic interventions for healthspan-limiting diseases.

Mitochodnrially targeted small peptide antioxidants

The second main class of mitochondrially-targeted antioxidants comprises small peptide molecules designed by Hazel Szeto and Peter Schiller (Zhao et al., 2003). These molecules do not require mitochondrial membrane potential for uptake into the mitochondrial inner membrane. The chemical characteristics of the amino acid side chains, including certain aromatic-cationic motifs, facilitate their cell permeability and mitochondrial membrane targeting (Zhao et al., 2005; Zhao et al., 2003; Zhao et al., 2004).

Two distinct SS peptides have been most studied, SS-31 and SS-20. The structure of SS-31 is D-Arg-Me_2Tyr-Lys-Phe-NH_2 while the structure of SS-20 is Phe-D-Arg-Phe-Lys-NH_2 (Cho et al., 2007a). The ROS scavenging activity of SS-31 is attributed to the dimethyltyrosine (dimethylTyr or Dmt) side chain. Lack of the Dmt side chain in SS-20 peptide renders it unable to scavenge ROS, but as we will discuss later in this section, SS-20 is not devoid of biological activity. As reviewed by Szeto (2008), as a class, the SS-peptides have numerous characteristics which lend easily to use as pharmaceutical interventions. They are small peptides which are readily synthesized and have been delivered by parental routes including IV, SC or IP. Despite being water soluble, they easily cross the blood brain barrier, but as yet, have poor enteral bioavailability, likely due to digestion by pancreatic enzymes in the small bowel. Despite the current lack of an enteral form of the SS-peptides, they have been successfully administered for weeks to months in rodent models.

SS-31 scavenges an array of mitochondrial ROSs that are reactive with the Dmt moiety including superoxide, peroxide, peroxynitrite and the hydroxyl radical (Zhao et al., 2005; Zhao

et al., 2003). In addition, the localization of SS-31 in the mitochondrial inner membrane as been shown to prevent cardiolipin oxidation with resulting protection from pro-apoptotic signals (Zhao et al., 2003).

The efficacy of SS-31 as an antioxidant has been demonstrated in an array of *in situ* and *in vivo* models and studies. In culture cells, SS-31 protects against a variety of exogenous oxidants including H_2O_2 (Petri et al., 2006; Whiteman et al., 2008; Zhao et al., 2005). Functional protection from endogenous ROS was recently demonstrated using a model of endothelial cells exposed to shear stresses (Han et al., 2009). The *in vivo* efficacy of SS-31 encompasses numerous physiological ROS challenges including ischemia-reperfusion heart and kidney, neuroprotection from ischemia-reperfusion and MPTP, as well as renal damage secondary to hydronephrosis (Cho et al., 2007a; Cho et al., 2007b; Mizuguchi et al., 2008; Song et al., 2005; Yang et al., 2009). Recently, SS-31 and MCAT expression were shown to maintain insulin sensitivity in rodents fed a high fat diet, as was discussed previously (Anderson et al., 2009).

An interesting addition to the developing SS peptide story is the *in vivo* effectiveness of the SS-20 peptide. As mentioned previously, the SS-20 peptide was developed without the antioxidant side chains as a control molecule (Zhao et al., 2004). However, SS-20 subsequently has demonstrated considerable *in vivo* activity by matching the protection of SS-31 in neuroprotection from MPTP although it cannot protect from cardiac ischemia-reperfusion injury (Cho et al., 2007a; Yang et al., 2009). SS-20 may work through other effects on the mitochondrial inner membrane or electron transport complexes and will serve as a powerful reagent for discerning the relative contribution of these mechanisms as compared to scavenging of mitochondrial ROS.

Mitochondrially-targeted, small molecule compound hold great promise. Their demonstrated ability to mimic the activities of endogenous antioxidant enzyme systems and subsequent physiological effects on pathways mediated by ROS facilitate their use in testing the causal involvement of ROS in aging and age-related diseases and facilitate translation to clinical use if found to be efficacious and if oral delivery can be made effective. While the potential for therapeutic use is an exciting development in the field of mitochondrial antioxidants, important information is pending regarding clear evidence of disease-modifying effects and, as with any pharmacological intervention, issues of compliance, tolerability of long-term treatment, and potential side effects.

The future of mitochondrially-targeted antioxidants

Next steps in the development and study of mitochondrially-targeted antioxidant will clearly build on the successes of the genetic models and small molecule compounds. More genetic models are needed to fill out the spectrum of information on different types of ROS, damage and signaling pathways, as well as tissue- or development-specific effects. These studies will continue to benefit from use of non-invasive *in vivo* techniques like echocardiography for collection of physiologically relevant longitudinal data. For each of these interventions, close examination is required of effects on other age-related diseases which are wide-spread in America's elderly and often difficult to prevent or reverse, such as cancer, sarcopenia, cognitive function, and kidney disease. Lastly, we need continued development of small molecule mitochondrially-targeted antioxidants both as possible disease-modifying interventions and as experimental reagents to facilitate continued interrogation of the free radical and mitochondrial hypotheses of aging.

The causality of mitochondrial oxidative stress in aging and age related disease, despite a nearly 40 year history of investigation, remains unclear. The studies in this review represent an exciting evolution of earlier hypotheses, the broader field of biogerontology and our growing

understanding of possible mechanisms of aging and disease. We predict that hypotheses will continue to evolve a focus on specific free radicals generated at distinctive sites within mitothondria and the exclusive targets of these radicals. The fundamental role of mitochondrial ROS in myriad cellular and pathological processes insures the significance of each discovery and the promise of connecting these processes to the larger phenomenon of aging.

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