Extension of Murine Life Span by Overexpression of Catalase Targeted to Mitochondria

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To determine the role of reactive oxygen species in mammalian longevity, we generated transgenic mice that overexpress human catalase localized to the peroxisome (PCAT), nucleus (NCAT), or mitochondrion (MCAT). Median and maximum lifespans were maximally increased (average 5 months, and 5.5 months, respectively) in MCAT animals. Cardiac pathology and cataract development were 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. These results support the free radical theory of aging and reinforce the importance of mitochondria as a source of these radicals.

A causative role for reactive oxygen species (ROS) in aging processes, referred to as the free radical theory of aging (*1*), proposes that ROS in biological systems attack molecules and cause the functional decline of organ systems that eventually lead to death. Accumulation of this damage over time is thought to result in pathologies associated with aging including arteriosclerosis, neoplasia, and cataracts (*2*). ROS are generated, in large part, from single electrons escaping the mitochondrial respiratory chain and reducing molecular oxygen to form the superoxide anion (O_2^{-}). Superoxide dismutase (SOD) converts O_2^{-} into hydrogen peroxide (H_2O_2) that then produces a highly reactive hydroxyl radical ($^{\cdot}OH$) in the presence of reduced metal atoms unless H_2O_2 is removed by the action of glutathione peroxidase or catalase.

The hypothesis that longevity can be enhanced by increasing antioxidant defenses has been controversial, based on contradictory findings in invertebrate models of aging. These include whether or not the overexpression of SOD or catalase enhances the lifespan of the fruit fly *Drosophila melanogaster* (*3–6*) and whether synthetic antioxidants extend the lifespan of the nematode *Caenorhabditis elegans* (*7–10*). Although there are an increasing number of long-lived mutant mouse models, most of these do not directly test

the free radical theory of aging. Overexpression of the antioxidant protein thioredoxin was reported to increased mean and maximum lifespan in a short-lived strain, although the identities of the specific agents that limited lifespan were not determined (*11*).

To determine the role of H_2O_2 in limiting mammalian lifespan, human catalase, normally localized in the peroxisome (PCAT), was targeted to the nucleus (NCAT) and mitochondrion (MCAT). Catalase activities in MCAT animals were elevated in heart and skeletal muscle of both founder lines (Fig. 1A and B) and in brain (Fig. 1C) of the 4033 founder line. Furthermore, catalase activity was 50-fold elevated in the cardiac mitochondrial fraction of MCAT animals, compared to their wild type (WT) littermates (Fig. 1D). Quantitative RT-PCR confirmed transgene expression in these tissues (fig. S1) (*12*). Endogenous catalase expression was similar between MCAT and WT animals, with the highest expression in liver, kidney, and lung (fig. S1). Confocal immunolocalization revealed that approximately 10-50% of cells in the MCAT heart expressed detectable levels of human catalase, co-localized with a mitochondrial marker in heart and fibroblast cultures from MCAT transgenic animals (Fig. 2). Human catalase was not detected in heart or fibroblasts from WT littermates. PCAT and NCAT gene products localized to peroxisomes and nuclei, respectively, as previously described (*13*).

To determine if the expression of PCAT, NCAT or MCAT could modulate lifespan, transgenic animals and WT littermates were maintained until death. PCAT animals showed a slight extension of median lifespan of 3 months (10%) and 3.5 months (13%) in the two founder lines compared to controls (Fig. 3A); this was significant only for the 2088 line (p = 0.02). Differences in maximal lifespan were not statistically significant. NCAT mice showed only 1 month (4%) and 3 month (11%) increases in median lifespan in the two founders; neither was significant (Fig. 3B).

Targeting catalase to the mitochondrion, however, afforded a 4.5 month (17%, $p < 0.0001$) and 5.5 month (21%, $p = 0.0002$) increase in median lifespans of founders 4403 and 4033, respectively (Fig. 3C). There was a similar extension of maximal lifespan: the 10% longest lived MCAT animals showed a 4.5 month longer median lifespan than WT littermates (both founders combined, $p = 0.001$). Increased lifespan was evident in both males ($p < 0.0001$) and females ($p = 0.0003$), without any statistically significant sex differences (fig. S2). The MCAT longevity data fits a Gompertz distribution (exponential increase in mortality rate with age) with parallel log mortality rates for MCAT and WT littermates (fig. S3), a result often interpreted as a delay in onset of aging. None of the transgenic lines showed a difference in weight or food consumption when compared with littermate controls (table S1) and there were no gross physical abnormalities.

Young (9-11 mo) and older (20-25 mo) MCAT and WT littermates were examined by histopathology. Little abnormality was seen in either group at 9-11 months of age. In older animals there was a trend toward reduced splenomegally and splenic lymphoid neoplasia in MCAT (1 of 21) vs. WT (4 of 24) mice, but this effect was not statistically significant. Cardiac pathology (sub-endocardial interstitial fibrosis, hyaline cytoplasmic change, vacuolization of cytoplasm, variable myocyte fiber size, hypercellularity, collapse of sarcomeres, mineralization, and arteriolosclerosis) was the most consistent difference between 20-25 mo MCAT and WT mice. These changes are also commonly observed in elderly human hearts, often in association with congestive heart failure (*14*); the latter has also been associated with functional abnormalities of mitochondria (*15*). The severity of pathology was graded on a score of 0 to 4 for a cross-sectional cohort of 21 MCAT and 20 WT mice age 20-25 months from both founder lines. The severity of arteriosclerosis was 1.29 on average for MCAT and 1.85 for WT ($p = 0.04$). The severity of cardiomyopathy was 1.19 for MCAT and 2.00 for WT ($p = 0.004$; $p = 0.002$ when combined with arteriosclerosis). This demonstrates the potential of the MCAT protein to protect the heart and suggests that these mice experience a prolonged healthspan as well as lifespan. The severity of cataracts, quantitated on a 4 point scale by slit-lamp examination, was reduced in 17 month old founder 4033 MCAT mice compared to age matched WT mice (1.5 ± 0.13 vs. 1.95 ± 0.13, respectively, $p = 0.003$), but not in founder 4403 compared to WT. However this trend became of borderline significance at 27 months ($p = 0.06$), and by 30 months age, both groups had similar cataract scores of ~2.5.

The ability of the MCAT protein to enhance protection of mitochondria from ROS was investigated by measuring aconitase activity in isolated heart mitochondria from 5, 19,

and 30-33 month old animals (Fig. 4A, B, and C). Aconitase is rapidly inactivated in H_2O_2-treated mitochondria isolated from WT hearts of all ages. This inactivation was significantly attenuated in MCAT heart mitochondria at all ages compared to controls, suggesting that these mitochondria eliminate H_2O_2 more effectively, and are thereby better protected from oxidative damage. The MCAT protein also decreased the mean level of H_2O_2 production from heart mitochondria 25% compared to WT animals (Fig. 4D), a significant difference ($p = 0.004$).

To determine whether MCAT overexpression could reduce oxidative damage to total DNA, 8-hydroxydeoxyguanosine (8-OHdG) was measured in DNA from skeletal muscle and heart. An age-related increase in 8-OHdG in skeletal muscle, but not heart, was observed in control animals, and MCAT mice were protected from this change (Fig. 4E, $p = 0.03$). Mitochondrial deletions associated with oxidative damage were measured as low molecular weight products by LX-PCR. These increased with age in both WT heart and skeletal muscle (*16*), however, a statistically significant decrease in the number of deletion products was noted in 21 month old MCAT skeletal muscle (Fig. 4F). A decrease was also detected in 30+ month MCAT skeletal muscle, and 21 month old MCAT heart, but neither reached statistical significance.

To examine the possibility that combined enhanced antioxidant defenses might provide further extension of lifespan in mammals, we bred hemizygous PCAT overexpressing animals to hemizygous SOD1 overexpressing animals (*21*). The double transgenic mice had an 18.5% extension of median lifespan compared to WT ($p < 0.0001$), and a 7% extension compared to PCAT littermates ($p = 0.036$), but without extension of maximum lifespan (Fig. 3D). There were no apparent deleterious phenotypic changes in these animals. It seems likely that SOD1 x MCAT or SOD2 x MCAT mice might exhibit an even greater extension of longevity, since both the combination of antioxidant enzymes chosen for enhancement and the subcellular localization appear to have profound effects on the lifespan extension phenotype.

The lifespan extension of MCAT mice (Fig. 3) was similar in magnitude to that resulting from knockout of the fat-specific insulin receptor (*17*), but less than that achieved by caloric restriction or dwarfism or that observed in other genetic models of delayed and decelerated aging (*18*). However, the effect of MCAT on lifespan is accomplished without apparent deleterious side effects and without disabling a major transduction pathway. While the MCAT longevity phenotype likely results from the direct beneficial effects of reduced oxidative stress in aging, it is also possible that indirect effects, such as stress response secondary to reduced intracellular H_2O_2 –dependent signaling, may also contribute to the longevity phenotype. The mosaic pattern of

catalase expression might also play a role in modulating the lifespan extension. Mosaicism may result from selection against cells expressing high catalase activities during early development since ROS may be an important mitogen (*19*) or. In addition, silencing of the CAG promoter-enhancer, and/or the progressive loss of transgene expression as the founder C3H alleles from the B6 (B6C3F1) hybrid embryos were diluted out through successive B6 back crosses may have reduced or modified MCAT expression (*20*). As a result, the observed MCAT protection against mitochondrial H_2O_2 toxicity, oxidative DNA damage and mtDNA deletion accumulation might have been much higher in the aging cohort mice than in the mice that were subsequently tested in biochemical assays. Aging cohort and cardiac pathology studies were performed on mice 2 to 4 generations after establishing the transgenic lines. Biochemical tests were done at generation 9 or later, when the genetic background was >99% B6, the mice had been moved to a new facility, and the lifespan extension phenotype appears to be diminished. Nonetheless, these results support the conclusion that mitochondrial ROS can be an important limiting factor in determining mammalian longevity and provide impetus for studies of new and combined antioxidant mouse models.

References and Notes

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Fig. 1. Catalase activity in MCAT animals. Catalase activities(*12*) (mean ± SEM) in control (open bars) and transgenic (closed bars) liver, kidney, heart and skeletal muscle whole tissue of two founder lines of MCAT mice, **(A)** founder 4033, and **(B)** founder 4403. Note differing ordinate scales. **(C)** Catalase activity in WT control and transgenic whole brain of these two lines (n=4 animals per group) **(D)** Catalase activities in the crude mitochondrial fraction (*12*) of 4033 transgenic and WT control heart (n=3 per group). * P < 0.05, ** P < 0.003, *** P <0.001.

Fig 2. Mitochondrial localization of human catalase. MCAT (**A**) and WT (**B**) mouse cardiac tissue (9 months old) stained for human catalase (green) and the mitochondrial marker cytochrome c (red) with a DAPI nuclear counterstain (blue). MCAT (**C**) and WT (**D**) mouse embryonic fibroblast cultures stained for human catalase (green) and the mitochondrial marker prohibitin (red) with a sytox green nuclear counterstain (blue). Scale bars indicate 20 microns.

Fig 3. Lifespan and catalase overexpression. Survival of transgenic animals and littermate pairs was analyzed for **(A)** PCAT **(B)** NCAT, and **(C)** MCAT, each for two independent founder lines. **(D)** shows the survival of all four genotypes resulting from a cross of hemizygous PCAT mice from line 2088 with hemizygous SOD1 overexpressing mice. The number of mice of each genotype is shown in parentheses.

P values of significant differences in mean lifespan between genotypes are indicated.

Fig 4. Aconitase activity, ROS production , and oxidative damage (mean ± SEM). Aconitase activity was measured in cardiac mitochondria from **(A)** 5-month, **(B)** 19-month, and **(C)** 30-33 month old MCAT and WT mice before and after treatment with 50μM H_2O_2 for 15 minutes at 0°C. N=6 for each group in A and B. In C, cardiac tissue was pooled from 3 animals of each genotype and measured in triplicate. When aconitase activity is expressed as a ratio of before vs. after H_2O_2 treatment, the differences between WT and MCAT are 3-fold at 5 months (p<0.002), 43-fold 19 months (p<0.0004) and >50-fold at 30-33 months (p<0.00005). **(D)** H_2O_2 production in cardiac mitochondria from 6 mo mice. **(E)** 8OHdG in skeletal muscle (black bars) and heart (white bars) of young and old mice. **(F)** LX-PCR results for genomic DNA derived from skeletal muscle and heart from 3 age groups. Significant difference between control (WT) and MCAT mice are shown. The numbers within the bars indicate the number of animals examined; in panel (E) 9 animals in 3 pools of 3 were examined in each category.

A

- ● PCAT 2058 (8)
- ○ WT 2058 (71)
- ■ PCAT 2088 (36)
- □ WT 2088 (22) } P=.02

B

- ● NCAT 885 (36)
- ○ WT 885 (46)
- ■ NCAT 900 (42)
- □ WT 900 (52)

C

- ● MCAT 4033 (20)
- ○ WT 4033 (44) } P=.0002
- ■ MCAT 4403 (42)
- □ WT 4403 (58) } P=.0001

D

- ■ PCAT+ SOD+ (62)
- ● PCAT+ SOD− (54)
- □ PCAT− SOD+ (56)
- ○ PCAT− SOD− (73)

P=.036
P=.0001
P=.013