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Science **308**, 1909 (2005);
DOI: 10.1126/science.1106653

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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, the nucleus, or mitochondria (MCAT). Median and maximum life spans were maximally increased (averages of 5 months and 5.5 months, respectively) in MCAT animals. Cardiac pathology and cataract development were delayed, oxidative damage was reduced, H₂O₂ production and H₂O₂-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 leads 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 (O₂⁻). Superoxide dismutase (SOD) converts O₂⁻ into hydrogen peroxide (H₂O₂) that then produces a highly reactive hydroxyl radical (-OH) in the presence of reduced metal atoms unless H₂O₂ is removed by the action of glutathione peroxidase or catalase.

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

of them do not directly test the free radical theory of aging. Overexpression of the antioxidant protein thioredoxin was reported to increase mean and maximum life span in a short-lived strain, although the identities of the specific agents that limited life span were not determined (11).

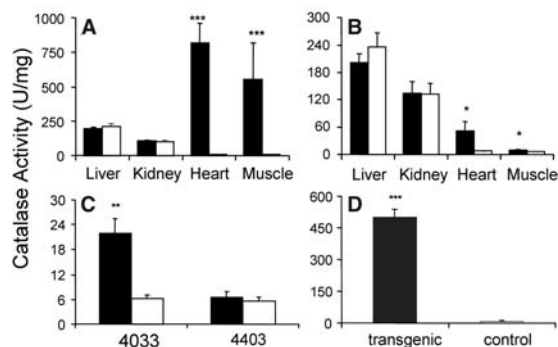
To determine the role of H₂O₂ in limiting mammalian life span, we targeted human catalase, normally localized in the peroxisome (PCAT), to the nucleus (NCAT) and mitochondria (MCAT). Catalase activities in MCAT animals were elevated in heart and skeletal muscle of both founder lines (Fig. 1, A and B) and in brain (Fig. 1C) of the 4033 founder line. Furthermore, catalase activity in the cardiac mitochondrial fraction of MCAT animals was 50 times higher than that in their wild-type littermates (Fig. 1D). Quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed transgene expression in these tissues (fig. S1) (12). Endogenous catalase expression was similar between MCAT and wild-type animals, with the highest expression in liver, kidney, and lung (fig.

S1). Confocal immunolocalization revealed that about 10 to 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 wild-type littermates. PCAT and NCAT gene products localized to peroxisomes and nuclei, respectively, as previously described (13).

To determine whether the expression of PCAT, NCAT, or MCAT could modulate life span, we maintained transgenic animals and wild-type littermates until death. PCAT animals showed a slight extension of median life span of 3 months (10%) and 3.5 months (13%) in the two founder lines compared with controls (Fig. 3A); this was significant only for the 2088 line ($P = 0.02$). Differences in maximal life span were not statistically significant. NCAT mice showed only 1-month (4%) and 3-month (11%) increases in median life span in the two founders; neither was significant (Fig. 3B). Targeting catalase to the mitochondria, however, afforded 4.5-month (17%, $P < 0.0001$) and 5.5-month (21%, $P = 0.0002$) increases in median life spans of founders 4403 and 4033, respectively (Fig. 3C). There was a similar extension of maximal life span: The 10% longest-lived MCAT animals showed a 4.5-month longer median life span than wild-type littermates (both founders combined, $P = 0.001$). Increased life span 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 fit a Gompertz distribution (exponential increase in mortality rate with age) with parallel log mortality rates for MCAT and wild-type 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 to littermate controls (table S1), and there were no gross physical abnormalities.

Young (9 to 11 months) and older (20 to 25 months) MCAT and wild-type littermates were examined by histopathology. Little ab-

Fig. 1. Catalase activity in MCAT animals. Catalase activities (12) (mean \pm SEM) in control (open) and transgenic (solid) 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 wild-type (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). Asterisk indicates $P < 0.05$; double asterisks, $P < 0.003$; and triple asterisks, $P < 0.001$.



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normality was seen in either group at 9 to 11 months of age. In older animals, there was a trend toward reduced splenomegally and splenic lymphoid neoplasia in MCAT (1 of 21) compared with wild-type (4 of 24) mice, but this effect was not statistically significant. Cardiac pathology (subendocardial interstitial fibrosis, hyaline cytoplasmic change, vacuolization of cytoplasm, variable myocyte fiber size, hypercellularity, collapse of sarcomeres, mineralization, and arteriosclerosis) was the most consistent difference between 20- to 25-month MCAT and wild-type 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 wild-type mice age 20 to 25 months from both founder lines. The severity of arteriosclerosis was 1.29 on average for MCAT and 1.85 for wild-type ($P = 0.04$). The severity of cardiomyopathy was 1.19 for MCAT and 2.00 for wild-type ($P = 0.004$; $P = 0.002$ when combined with arteriosclerosis). This demonstrates the potential of the MCAT protein to protect the heart and suggests that these mice experience a prolonged health span as well as life span. The severity of cataracts, quantitated on a four-point scale by slit-lamp examination, was reduced in 17-month-old founder 4033 MCAT mice compared with age-matched wild-type mice (1.5 ± 0.13 and 1.95 ± 0.13 , respectively, $P = 0.003$) but not in founder 4403 compared with wild-type. However, this trend became of borderline significance at 27 months ($P = 0.06$), and by the age of 30 months 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- to 33-month-old animals (Fig. 4, A, B, and C). Aconitase is rapidly inactivated in H_2O_2 -treated mitochondria isolated from wild-type 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 mean H_2O_2 production from heart mitochondria 25% compared with wild-type animals (Fig. 4D), a significant difference ($P = 0.004$).

To determine whether MCAT overexpression could reduce oxidative damage to total DNA, we measured 8-hydroxydeoxyguanosine (8-OHdG) 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 long-extension

polymerase chain reaction (LX-PCR). These increased with age in both wild-type heart and skeletal muscle (16); however, a statis-

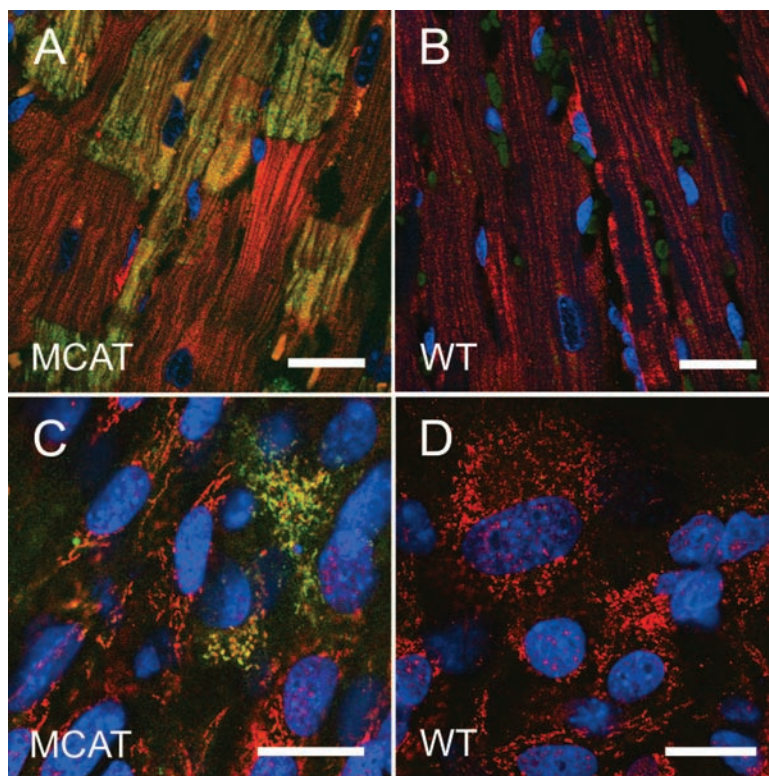


Fig. 2. Mitochondrial localization of human catalase. MCAT (A) and WT (B) mouse cardiac tissue (9 months old) stained for human catalase (green) and the mitochondrial marker cytochrome c (red) with a 4',6'-diamidino-2-phenylindole (DAPI) nuclear counterstain (blue). MCAT (C) and WT (D) mouse embryonic fibroblast cultures stained for human catalase (green) and the mitochondrial marker prohibitin (red) with a sytox green nuclear counterstain (blue). Scale bars indicate 20 μ m.

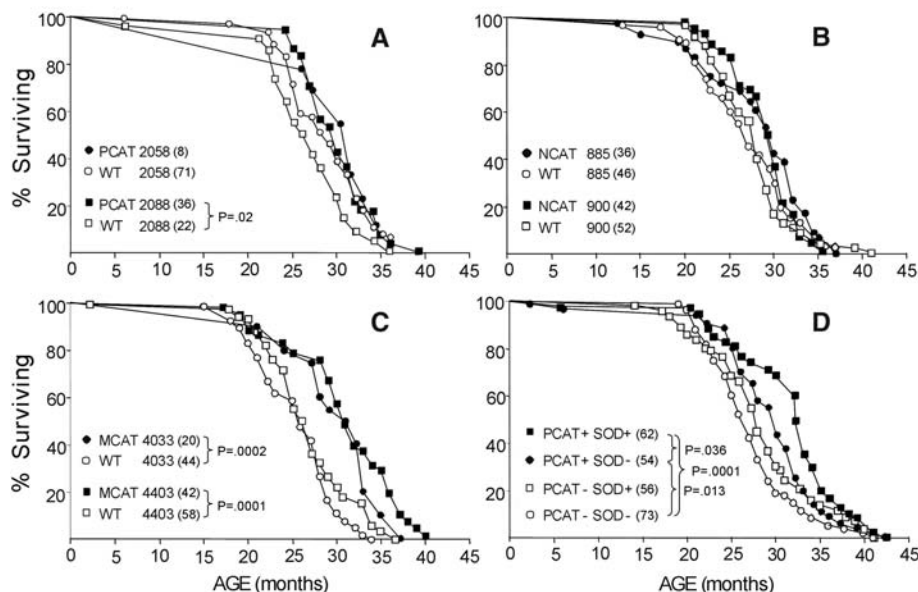


Fig. 3. Life span and catalase overexpression. Survival of transgenic animals and littermate pairs was analyzed for (A) PCAT (B) NCAT, and (C) MCAT, each for two independent founder lines. (D) shows the survival of all four genotypes resulting from a cross of hemizygous PCAT mice from line 2088 with hemizygous SOD1-overexpressing mice. The number of mice of each genotype is shown in parentheses. P values of significant differences in mean life span between genotypes are indicated.

tically 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 life span in mammals, we bred hemizygous PCAT-overexpressing animals to hemizygous SOD1-overexpressing animals (17). The double transgenic mice had an 18.5% extension of median life span compared with wild-type ($P < 0.0001$) and a 7% extension compared with PCAT littermates ($P = 0.036$), but without extension of maximum life span (Fig. 3D). There were no apparent deleterious phenotypic changes in these animals. It seems likely that SOD1 \times MCAT or SOD2 \times MCAT mice might exhibit an even greater extension of longevity, because both the combination of antioxidant enzymes chosen for enhancement and the subcellular localization appear to have profound effects on the life span extension phenotype.

The life span extension of MCAT mice (Fig. 3) was similar in magnitude to that resulting from knockout of the fat-specific insulin receptor (18) but less than that achieved by caloric restriction or dwarfism or that observed in other genetic models of delayed and decelerated aging (19). However, the effect of MCAT on life span is accomplished without apparent deleterious side effects and without disabling a major transduction pathway. Although 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 a stress response secondary to reduced intracellular H₂O₂-dependent signaling, may also contribute to the longevity phenotype. The mosaic pattern of catalase expression might also play a role in modulating the life span extension. Mosaicism may result from selection against cells expressing high catalase activities during early development because ROS may be an important mitogen (20). In addition, silencing of the CAG promoter-enhancer and/or the progressive loss of trans-

gene 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 (21). As a result, the observed MCAT protection against mitochondrial H₂O₂ toxicity, oxidative DNA damage, and mitochondrial DNA 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 two to four generations after establishing the transgenic lines. Biochemical tests were done at generation 9 or later, when the genetic background was >99% B6 and the mice had been moved to a new facility, and the life span 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.

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22. We thank L. Loeb, B. Preston, and E. Ruiz-Pesini for insightful comments; S. Chen, S. Tsang, S. Knoblauch, R. Mangalindan, and N. Hudson for technical contributions; and C. Epstein for SOD1-overexpressing animals. Supported by NIH grants AG001751, ES07033, and AG13280.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1106653/DC1
 Materials and Methods
 Figs. S1 to S3
 Table S1
 References

22 October 2004; accepted 15 April 2005
 Published online 5 May 2005;
 10.1126/science.1106653
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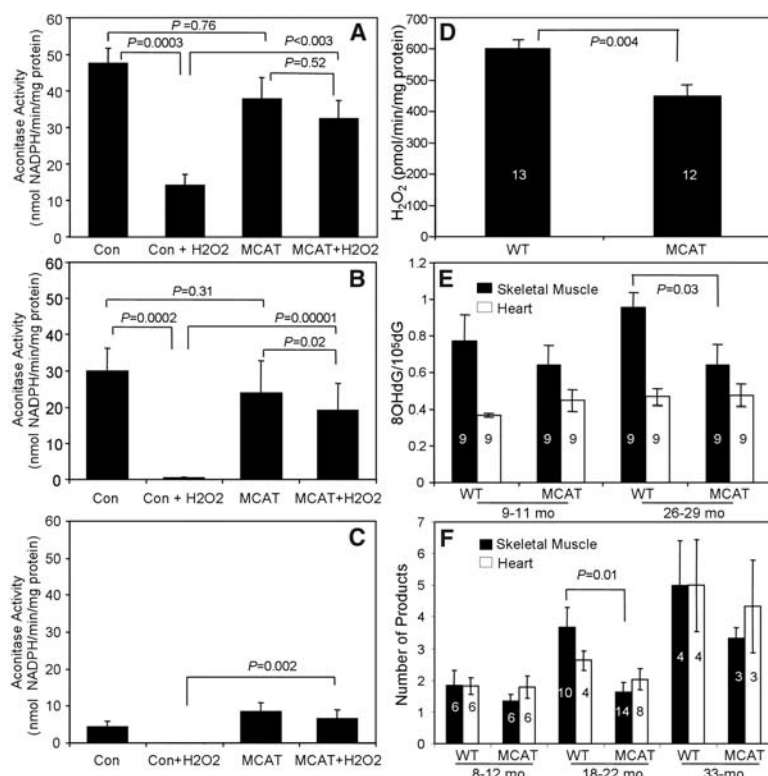


Fig. 4. Aconitase activity, ROS production, and oxidative damage (mean \pm SEM). Aconitase activity was measured in cardiac mitochondria from (A) 5-month, (B) 19-month, and (C) 30- to 33-month-old MCAT and WT mice before and after treatment with 50 μ M H₂O₂ for 15 minutes at 0°C. $N = 6$ for each group in (A) and (B). In (C), cardiac tissue was pooled from three animals of each genotype and measured in triplicate. When aconitase activity is expressed as a ratio of before versus after H₂O₂ treatment, the differences between WT and MCAT are 3-fold at 5 months ($P < 0.002$), 43-fold at 19 months ($P < 0.0004$), and >50-fold at 30 to 33 months ($P < 0.00005$). (D) H₂O₂ production in cardiac mitochondria from 6-month-old mice. (E) 8OHdG in skeletal muscle (black) and heart (white) of young and old mice. (F) LX-PCR results for genomic DNA derived from skeletal muscle and heart from three age groups. Significant differences between control (WT) and MCAT mice are shown. The numbers within the bars indicate the number of animals examined; in (E), nine animals in three pools of three were examined in each category.