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A carboxyfullerene SOD mimetic improves cognition and extends the lifespan of mice

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Abstract

In lower organisms, such as *Caenorhabditis elegans* and *Drosophila*, many genes identified as key regulators of aging are involved in either detoxification of reactive oxygen species or the cellular response to oxidatively-damaged macromolecules. Transgenic mice have been generated to study these genes in mammalian aging, but have not in general exhibited the expected lifespan extension or beneficial behavioral effects, possibly reflecting compensatory changes during development. We administered a small-molecule synthetic enzyme superoxide dismutase (SOD) mimetic to wild-type (*i.e.* non-transgenic, non-senescence accelerated) mice starting at middle age. Chronic treatment not only reduced age-associated oxidative stress and mitochondrial radical production, but significantly extended lifespan. Treated mice also exhibited improved performance on the Morris water maze learning and memory task. This is to our knowledge the first demonstration that an administered antioxidant with mitochondrial activity and nervous system penetration not only increases lifespan, but rescues age-related cognitive impairment in mammals. SOD mimetics with such characteristics may provide unique complements to genetic strategies to study the contribution of oxidative processes to nervous system aging.

Keywords: Aging; Lifespan; Superoxide dismutase mimetic; Free radical; Oxidative stress; Mitochondria; Antioxidants; EPR; Carboxyfullerene

1. Introduction

Aging appears to be determined by a combination of genetic and environmental factors. Elegant work in *Caenorhabditis elegans* has identified a number of genes including *clk-1*, *age-1*, *daf-2*, *daf-16* [11] and *Sir2* [42] that are crucial to aging. Age-1, daf-2 and daf-16, which are associated with the insulin-dependent signaling, are specifically involved in the response to oxidative stress, thus creating a genetic link between stress resistance and longevity. For example, age-1 mutants, which have a 65% increase in lifespan, exhibit age-dependent elevations in the activities of both superoxide dismutase and catalase [28]. Daf-2 mutants live twice as long as wild-type worms and demonstrate increased resistance to oxidative stress, a response that is dependent on daf-16 [11]. Both age-1 and daf-2 suppress daf-16 phosphorylation, allowing its subsequent translocation into the nucleus where it induces expression of an entire cassette of defense protein genes, including several antioxidant enzymes. Such genetic findings complement an already extensive body of literature showing accumulation of oxidatively modified macromolecules, especially mitochondrial DNA (mtDNA), during aging in an array of taxa including fish, flies, worms and mammals [32]. One scheme proposed to integrate these observations is the *mitochondrial free radical theory of aging* [14], which postulates that free radical

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reactions – inherent to normal biology – result in increasing cell damage and eventual cellular senescence during aging, and that mitochondria are specifically both a source and a target of free radical-mediated damage. While appealing and indirectly supported, data from mammals has been less than conclusive. Mice that overexpress antioxidant enzymes have not, with one notable exception, been more long-lived than their non-transgenic littermates. That one exception was a recent report indicating that mice with mitochondrially-targeted expression of catalase had a significant increase in lifespan [38]. While transgenic mice have been powerful tools to demonstrate the importance of other pathways, such as the insulin signaling pathway (*e.g.* IGF-1R, Ghrhr, $p66^{shc}$, Sirt1; for review, see [29,27,13]) to aging and longevity [27,29,35], their use for studies of ROS in aging may be limited due to compensatory effects that can clearly occur during early development. Such compensation may mask the natural regulation of antioxidant systems that occurs during aging and alter other aspects of physiology including reproductive fecundity. For example, ablation of germ line cells increases lifespan of *C. elegans* by 60% [18]. Our study initiated treatment of non-transgenic C57BL/6 mice with a mitochondrially-active SOD mimetic [1] at 12 months of age, after both development and reproduction are complete, thereby avoiding concerns about effects that are unrelated to the aging process, itself. In this study, we evaluated lifespan, immune system function, mitochondrial activity and behavioral and cognitive performance in chronically treated mice, with the goal of determining whether a single well-defined agent (as opposed to calorie restriction, a robust but highly complex intervention) could modify aging and age-related pathology in mammals.

2. Materials and methods

2.1. Mouse care, diets and treatment protocols

Mice were purchased from Taconic Farms (Germantown, NY) at 10 M of age and housed in a pathogen-free facility under conventional conditions. Individually housed animals were maintained in 12-h light/12-h dark light cycles at 24 °C with relative humidity of 50%. Upon arrival, all mice were provided NIH-31 chow *ad libitum* (average intake 15 kcal/day) as described [44], and were randomly assigned, with no selection for pre-existing disease or pathology, to various survival cohorts or for pre-morbid biochemical studies. Mice in survival studies were allowed to die of natural causes and the date of death recorded by animal facility staff. Treatment of mice with the C_3 (*e*,*e*,*e*-$C_{60}(C(COOH_2))_3$) compound (10 mg/kg/day) was initiated at 12 M of age by placing C_3 in the drinking water. Control mice received color-matched dilute food coloring in their water. C_3-treated and control mice were fed NIH-31 chow *ad libitum* throughout the study. We have previously determined that C_3 can cross the blood–brain barrier (Dugan, unpublished).

2.2. Synthesis of the C_3 compound

C_3 (*e*,*e*,*e*-$C_{60}(C(COOH_2))_3$) was synthesized as described previously [1,9,31]. Purity was confirmed by liquid chromatography–mass spectroscopy (LCMS) and LC-diode array absorbance spectroscopy [31].

2.3. Evaluation of dihydroethidium oxidation

To evaluate cell-specific ROS formation, we measured dihydroethidium (DHE) oxidation as described by Quick and Dugan [37]. Briefly, mice were given a series of two 27 mg/kg ip injections of DHE, separated by 30 min, and sacrificed 18 h following the initial injection. Mice were anesthetized and perfusion fixed with paraformaldehyde. Following removal, brains were post-fixed and sectioned for examination by confocal microscopy. Ethidium fluorescence (Ex λ 488 nm, Em λ >560 nm) was visualized on a Zeiss LSM510 META confocal laser scanning microscope equipped with 488 and 543 nm laser lines using a 63× C-Apochromat (NA 1.2) water immersion objective. Excitation and emission settings were determined to detect the superoxide-mediated intracellular oxidation of DHE [50]. Image processing was performed using MetaMorph (Universal Imaging) software to quantify average cytosolic pixel intensity. Greater than 60 cells per brain region were examined per animal and averaged which were imaged from four brain sections.

2.4. Isolation of brain mitochondria

Mitochondria from mouse brain were isolated by homogenization and differential centrifugation according to a protocol developed in our lab [24] which is a modification of a reported method [39]. The protocol was approved by the animal care committee of the University of California, San Diego. Mice were anesthetized with Halothane and immediately perfused with cold PBS. The entire cerebral cortex was removed and rinsed twice by ice-cold isolation buffer (IB; 0.32 sucrose, 10 mM Tris–HCl buffer, pH 7.2 and 1 mM EDTA) and the wet-weight was recorded after gentle blotting. After mincing in 2 mL of IB, the tissue was homogenized in the same buffer (10 mL/g) and centrifuged at 1330 × *g*. The supernatant was saved and the pellet is re-homogenized in the one-half volume of the original IB used and centrifuged at 1330 × *g* for 3 min. Both supernatants were pooled and spun at 21,200 × *g* for 10 min and the pellet was then re-suspended in 8 mL/g of initial tissue weight. To the suspension, 4.6 mL of 40% percoll/g of tissue was added and the mixture was then loaded over 23 and 40% percoll gradient. The mixture was then centrifuged at 30,700 × *g* for 5 min and the fraction accumulated at the interface between the two lower layers was collected using a syringe. The collected fraction was then diluted 1:4 with IB and centrifuged at 16,700 × *g* for 10 min. After re-suspension in IB (5 mL/g tissue) containing 1 mg/mL bovine serum albumin (BSA), the mixture was centrifuged at 6900 × *g* for 10 min. The brown pellet con-

taining isolated mitochondria was re-suspended in 500 μL IB using 1 mL Dounce homogenizer. Mitochondria protein concentration was determined by the Lowry method using BSA as standard and the purity of the isolated mitochondria was confirmed by enrichment of cytochrome oxidase activity.

2.5. EPR experiments

We employed a Bruker e-scan benchtop EPR spectrometer for these studies. The mixture was injected into the EPR cavity of Bruker e-scan benchtop spectrometer via a Teflon tube with ~0.4 mm i.d. The EPR setting were, receiver gain 1×10^3, scan width 200 G centered at 3484.9 G, modulation amplitude 2 or 4 G, time constant 5.16 ms, modulation frequency 86 kHz, microwave power 5.04 mW, 5.24-s sweep time, and the spectrometer's operating frequency 9.784 GHz. Each spectrum was the average of 100-times accumulations.

A set of control experiments were carried out to assign and calibrate the EPR signal from DEPMPO-superoxide using superoxide generated by hypoxanthine/xanthine oxidase metabolism. Assignment of the observed signals from mitochondria was confirmed through computer-assisted spectral simulation using the WinSim software (http://epr.niehs.nih.gov/pest.html). In most cases, a mixture of signals due to DEPMPO-OOH and DEPMPO-OH adducts was detected but the complete removal of these signals upon the inclusion of SOD confirmed that superoxide radical was the exclusive source of the observed EPR signals.

EPR spectra were observed upon the incubation at 37 °C for one hour of 1–2 mg protein/mL brain mitochondria isolated from young (3 months) or old (25 months) mice. In each incubation, 10 mM pyruvate, 5 mM malate and 1 mM ADP were included. In some experiments, 50 or 100 U/mL SOD was included to confirm that the observed signal was derived from superoxide. EPR intensities were normalized for the protein content and are shown as mean ± standard error. Mitochondrial viability was determined by assessing O_2 consumption as described below.

2.6. Oxygen consumption experiments

Oxygen consumption was assessed using a Hansatech Oxygraph unit employing a Clark-type O_2 electrode (Hansatech Instruments, UK). Reaction mixtures in the appropriate buffer, typically containing 250–320 μM oxygen, were introduced into a closed glass compartment above the electrode system and continuously stirred at ambient temperature.

In order to confirm viability of our isolations, parts of the isolated mitochondria suspensions were used to monitor the oxygen consumption in the presence of malate/pyruvate (state 4) and after the further addition of ADP (state 3). The respiratory control ratios (RCR) of (state 3/state 4) for each group of animals were calculated. Values of RCR were in the range from 4 to 6 which indicates tightly coupled mitochondrial respiration.

2.7. Morris water maze

Spatial learning and memory capabilities were evaluated using a modified Morris water navigation protocol similar to our previously published methods [16,49]. However, our protocol was modeled after one developed by Morris and colleagues [7] which has been shown to be sensitive for detecting age-related spatial learning deficits in transgenic mice that overexpress human mutant amyloid precursor protein. The protocol included three different types of trials: cued (visible platform; 60-s duration), spatial (submerged and not visible platform; 60-s duration) and probe (platform removed; 30-s duration). All trials were conducted in a round pool (118 cm i.d.) of opaque water and videotaped, while swim paths were tracked and recorded by a computerized system (Polytrack, San Diego Instruments, San Diego, CA), which calculated escape path length and latency. Mice were first trained on the cued condition, which included swimming to a submerged platform that had a red tennis ball fixed on top of a rod marking its location, to determine if non-associative factors (*e.g.* sensorimotor or visual disturbances or alterations in motivation) were likely to affect acquisition performance during subsequent spatial trials. Mice received two blocks of two trials each (60-s intertrial interval (ITI) and 2 h between blocks) for 2 consecutive days where the platform was moved to a new location for each trial with very few distal cues present. Three days later the mice were trained during spatial trials to learn the location of a submerged platform which was not apparent since the rod and tennis ball were removed. Multiple distinct spatial cues were positioned around the room for the place trials to facilitate association of cues with the submerged platform location. During acquisition training in the spatial condition, the mice were given two blocks of two trials per day (60-s maximum; 60-s ITI) for 5 days per week over a 3-week period with each block being separated by approximately 2 h. The spatial condition was different from the standard place trials procedure in that the submerged platform remained in the same location for all trials within a given week, but it was moved to a new location for each of the next 2 weeks. Morris and colleagues [7] have hypothesized that changing platform locations induces potential interference for encoding future locations, thus introducing an episodic memory-like component and increasing the memory demands of the task. Two probe trials were administered on the 5th day of each of the 3 weeks of the spatial condition. One probe trial was administered first before conducting any spatial trials, thus representing a 24-h retention interval since the last place trial. A 2nd probe trial was also administered 1 h after the last probe trial on day 5, thus representing a 1-h retention interval. During the 60-s probe trial, the escape platform was removed and a mouse was placed in the quadrant diagonally opposite from the previous platform location and its search behavior was quantified. Time spent in the quadrant where the platform had been, and the number of crossings made over the previous platform location (platform crossings), were recorded.

2.8. Statistical analysis

This was a prospectively designed longitudinal experimental study. Sample size determination for survival cohorts was based on the statistical comparison of the survival curves between the control group and the C_3-treatment group [47]. As previous studies such as that by Turturro et al. [44] suggested a guarantee time in the survival distributions, the sample size determination was based on the log-rank test applied to two distributions after this guarantee time [46]. Survival distributions for C_3-treated and control mice were estimated by the non-parametric Kaplan–Meier Product-Limit estimator [20]. Cox's proportional hazards model was used to estimate and test the hazard ratio of death between the two mice groups [41]. The assumption of proportional hazards was also examined and verified by a test through the incorporation of time-dependent covariates [41].

Statistical significance of DHE oxidation, EPR spectra, lymphocyte populations, mitochondrial electron transport chain activities was determined using ANOVA followed by Student–Newman–Keuls. Analysis of variance (ANOVA) models typically containing one between-subjects variable (Groups) and one within-subjects (repeated measures) variable (*e.g.* Blocks of Trials) were used to analyze the behavioral data. The Huynh–Feldt adjustment of alpha levels was used for all within-subjects effects containing more than two levels to protect against violations of sphericity/compound symmetry assumptions, and Bonferroni corrected "*p*" values were used when multiple comparisons were conducted.

3. Results

3.1. Extension of lifespan in mice treated with a C_{60} fullerene SOD mimetic

Although we were interested in determining effects of treatment on CNS function during aging, we first determined to what extent C_3, a mitochondrially active SOD mimetic, modified the aging process in a global sense, by evaluating lifespan. Normal C57BL6 mice, received C_3, a small C_{60} fullerene compound (Fig. 1) with SOD mimetic properties [1] starting at 12 months of age. This fullerene derivative has previously been shown to be neuroprotective in acute and chronic injury models [10] and to increase survival of *Sod2*$^{-/-}$ mice which lack the mitochondrial MnSOD (*Sod2*) gene [1]. Survival analysis was performed on an uncensored (Fig. 2a) and censored (Fig. 2b) cohort of animals. The survival distributions for C_3-treated and control mice were estimated by the non-parametric Kaplan–Meier Product-Limit estimator (Fig. 2a and b) and compared by a log-rank test [20]. For the uncensored cohort, mice with C_3 treatment ($n=46$; 23 males, 23 females) had a lower rate of death over time (log-rank test = 8.33, $p=0.004$) compared to control mice ($n=45$; 24 males, 21 females) (Fig. 2a). The estimated median lifespan for the C_3-treated mice was 27.5 months and

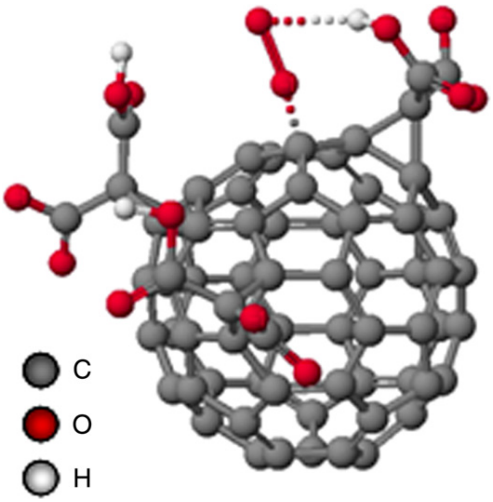

Fig. 1. Structure of the C_3 regioisomer of Tris–malonic acid C_{60} fullerene derivative (*e*,*e*,*e*-$C_{60}(C(COOH)_2)_3$). A superoxide radical anion is shown at top.

that for control mice was 24 months. C_3 treatment shifted the mean survival from 24.8 ± 0.7 months for controls to 27.6 ± 0.6 months (mean ± S.E.M.) for C_3-treated mice, an increase of 11%. Under the assumption of proportional hazards between the two groups which was verified by a test incorporating time-dependent covariates [41], the estimated hazard ratio of death between the C_3-treated mice and the control mice was 0.561 with a 95% confidence interval ranging from 0.364 to 0.864. To further confirm these findings, we examined our mixed sex experimental groups by another Cox proportional hazards model [20], which controls for the gender effect by treating it as strata (hazard ratio of death between the C_3-treated mice and the control mice = 0.569, $p=0.011$). C_3-treatment also increased the longest-lived 5%, which includes three animals per group (Control, 32, 32, 32 months; C_3-treated, 35, 35, 36 months).

Additionally, we combined survival data into a censored curve (Fig. 2b) which includes data from Fig. 2a and two additional censored cohorts of animals utilized for biochemical studies. The lifespan for 38 of the 123 control mice (male, $n=62$; female, $n=61$) and 46 of the 115 C_3-treated mice (male, $n=56$; female, $n=59$) were censored. As with the uncensored survival data, survival distributions were estimated by non-parametric Kaplan–Meier Product-Limit estimator and compared by a log-rank test [20]. Mice treated with C_3 had a lower rate of death over time (log-rank test statistic = 11.4, $p=0.0007$) compared to control mice. The estimated mean lifespan for C_3-treated was 27.4 ± 0.5 months (mean ± S.E.M.) and 25.1 ± 0.4 months for control mice. Under the assumption of the proportional hazards between the treated mice and the control groups which was verified by a test through the incorporation of the time-dependent covariates [41], the estimated hazard ratio of death between C_3-treated mice and the control mice was 0.590 with a 95% confidence interval ranging from 0.424 to 0.820. These find-

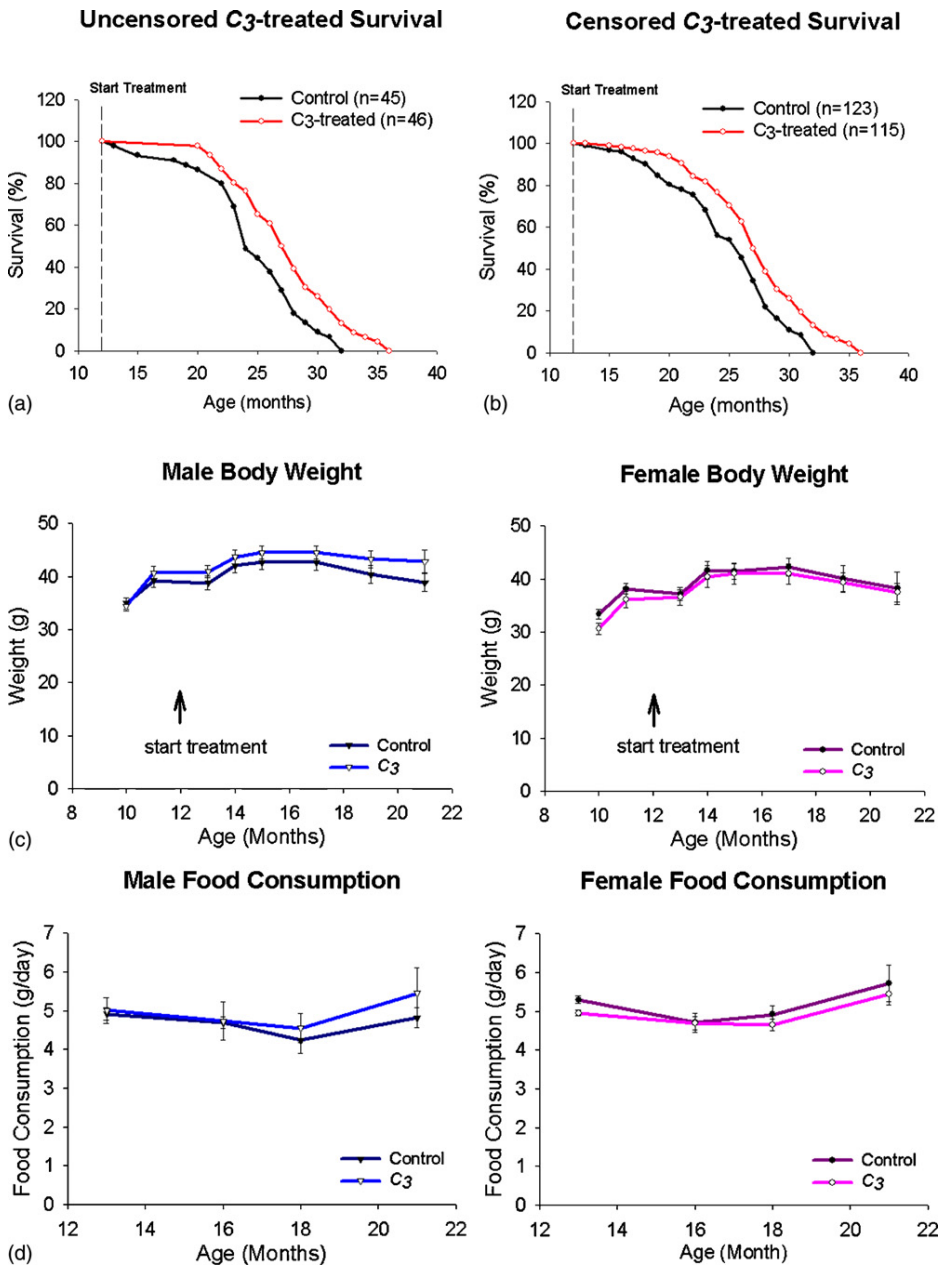

Fig. 2. Lifespan curves for C_3-treated and control mice. Treatment of mice with C_3 or color-matched dilute food coloring in their drinking water was initiated at 12 months of age, and continued until death. (a) Administration of C_3 increased mean survival by 11% and extended maximal survival from 32 months (control) to 36 months (C_3) in an uncensored cohort of mice. (b) Survival data from panel (a) combined with two additional cohorts of mice some of which were utilized for biochemical analysis, and thus censored. (c) Weights of C_3-treated mice (males: $n=14$; females: $n=11$) were not statistically different from age-matched controls (males: $n=15$; females: $n=9$). (d) Food consumption was also not statistically different between C_3-treated mice and controls ($n=5$ each sex). Body weight and food consumption values are mean ± S.E.M.

ings were further confirmed by the analysis based on another Cox proportional hazards model [20], which controlled for the gender effect by treating it as a strata (hazard ratio of death between C_3-treated mice and the control mice was 0.588, $p=0.0016$). Calorie restriction has been reported to increase lifespan by two mechanisms; decreasing the incidence of fatal diseases (*e.g.* cancer) and by slowing *the rate of aging*. Changes in the maximal lifespan, which is encompassed in the Cox proportional hazards model, serve as an indicator of the rate of aging. The observation that C_3 treatment decreased the hazard rate of death suggests that C_3, like calorie restriction, may slow the rate of aging.

To exclude the possibility that C_3 treatment enhanced lifespan indirectly by producing calorie restriction through

altered feeding, we measured food consumption and body weight in a random subset of mice included in the longevity study. No significant differences in weights between C_3-treated mice and age-matched controls were observed (Fig. 2c). We also compared food intake of mice in both groups and found no significant differences at any age (Fig. 2d) indicating that C_3 treatment did not result in secondary calorie restriction. Together, these data indicate that treatment with a superoxide dismutase mimetic which is able to target mitochondrial superoxide can increase lifespan in mammals. Our findings extend previous work in *C. elegans* showing that a chemically different class of SOD mimetics, which contain manganese as the active moiety, enhanced lifespan [34], although extension of lifespan in *C. elegans* using these agents has not been universally observed [22,23].

3.2. Attenuation of age-associated increases in oxidative stress by C_3

Numerous studies have shown age-related increases in oxidative damage to macromolecules (lipid, protein and DNA) in a variety of organisms including humans [40]. To determine if C_3 affects age-associated increase in free radical levels, we examined levels of dihydroethidium (DHE) oxidation in brain from young, old and old C_3-treated mice. Since DHE is selectively oxidized by superoxide radical, systemic administration of DHE and uptake of the dye into brain allows for the *in vivo* detection of the level of superoxide [4,5,37,50]. In addition, imaging of DHE oxidation by confocal microscopy permits cell-specific detection of superoxide generation. Representative confocal microscopy images of

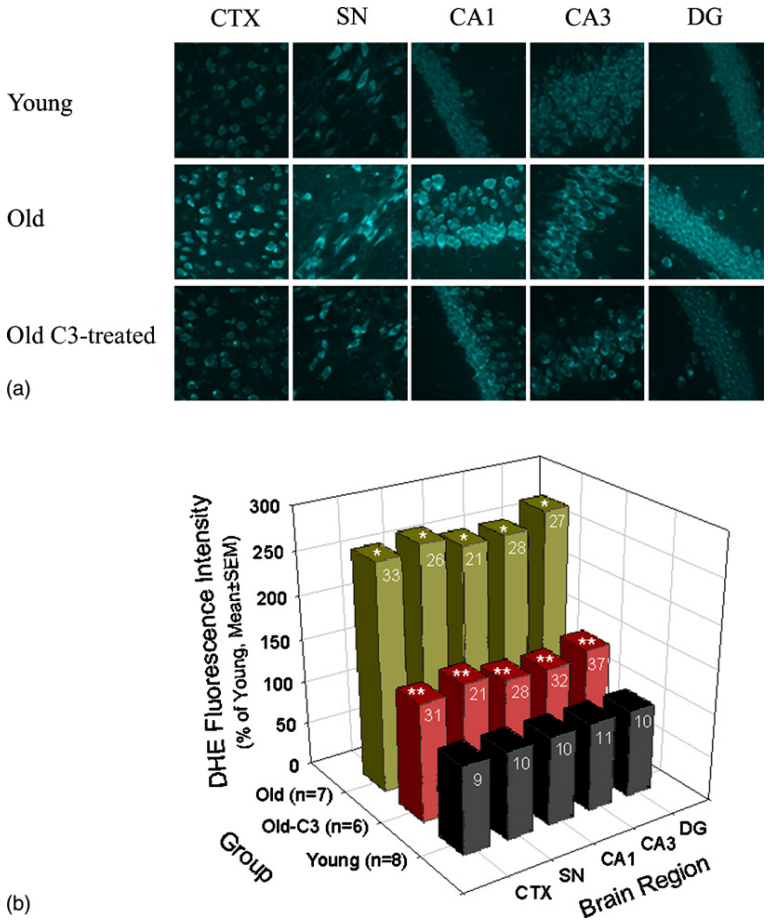

Fig. 3. Reduction of age-associated brain oxidative stress by C_3. Mice were placed on the same treatment regimens as those used for the longevity cohorts. Young (4 months, $n=8$), old (24 months, $n=7$) and old C_3-treated (24 months, $n=6$) groups were analyzed. Superoxide-mediated oxidative stress was measured by a systemically administered dye, dihydroethidium (DHE) [37]. (a) Confocal microscopy images of fixed brain slices superoxide-mediated DHE oxidation, shown in cyan. (b) Quantification of DHE oxidation in cortex (CTX), substantia nigra (SN) and CA1, CA3 and dentate gyrus (DG) regions of the hippocampus. Values are mean ± S.E.M.; S.E.M. is represented by numbers on the face of each bar. $^{*}p<0.05$ by ANOVA and Student–Neuman–Keuls *post hoc* analysis for young *vs.* old. $^{**}p<0.05$ by ANOVA and S–N–K *post hoc* analysis for old *vs.* old-C_3.

fixed brain slices are included in Fig. 3a, with superoxide-mediated DHE oxidation shown in cyan. Quantification of DHE oxidation within specific brain regions (Fig. 3b) showed significant increases in oxidized DHE in all brain regions measured with age (young, 4 months, $n=8$ *versus* old, 24 months, $n=7$) and a reduction by chronic C_3 treatment (old, 24 months, $n=7$ *versus* old-C_3, 24 months, $n=6$). Old control mice showed a 165% increase in fluorescence due to DHE oxidation *versus* young mice. Old C_3-treated mice, on the other hand, had only a 37% increase in fluorescence. These data indicate that C_3 treatment reduced age-associated elevations of superoxide as detected by DHE oxidation, decreasing general cellular oxidative stress in the specific brain regions examined. It would have been useful to carry out parallel experiments with a structurally similar fullerene compound that lacked SOD activity to confirm that the SOD activity of C_3 is important to its anti-aging affects, but it was not possible to produce the necessary quantity of such a compound needed when the study was started.

3.3. Reversal of mitochondrial free radical production by C_3

Mitochondria are responsible for the majority of O_2 consumption of respiring cells, and are thus the major cellular source of ROS. At present, direct measurements of background superoxide production by mitochondria, *i.e.* without the use of electron transport chain inhibitors, in relation to age has not been reported. To address this deficiency, we carried out a spin trapping EPR (electron paramagnetic resonance) study using 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) to monitor alterations in superoxide production occurring during aging in mice, and the effectiveness of C_3 to modulate these changes (Fig. 4). EPR spectra were captured from intact brain mitochondria from young (3 months, $n=14$), old (24 months, $n=17$) and old-C_3 (28 months, $n=11$) mice. Representative EPR spectra are shown in Fig. 4a. Quantification of spectra (Fig. 4b) showed a significant increase in the superoxide-mediated signal from isolated brain mitochondria with age and a significant attenuation of this increase in the chronically C_3-treated mice. To confirm that the increased EPR signal in old mice was derived from superoxide, SOD (100 U/mL) was included in certain experiments and abolished the EPR signal (Fig. 4a, old + SOD), indicating that it is superoxide-dependent.

3.4. Effects of SOD mimetic, C_3, on age-related loss of spatial learning and memory

The effects of chronic antioxidant therapy on age-associated loss of spatial learning and memory was tested using the Morris water maze [8,17]. Young (5 months, $n=23$), old (23–26 months, $n=18$) and old C_3-treated (23–26 months, $n=23$) mice were trained on the cued trials (Fig. 5a). Analysis of the cued trials data (Fig. 5a) showed that there was a significant main effect of Group for path length

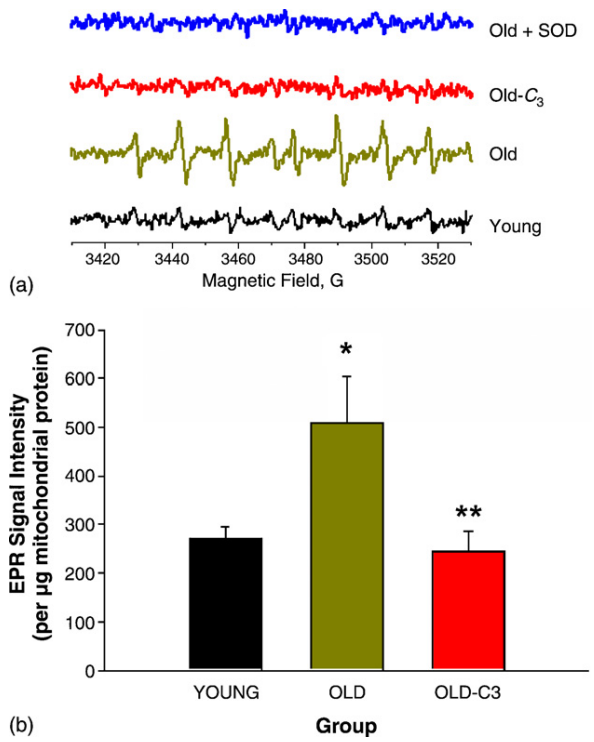

Fig. 4. Age-dependent increase in superoxide production by mouse brain mitochondria and reversal by C_3 treatment. (a) EPR spectra observed upon the incubation of isolated brain mitochondria for 1 h at 37 °C from 3-month-old (Young), 24-month-old (Old) and 24-month-old C_3-treated (Old-C_3) mice. Top (Old + SOD) trace shows sample from an old mouse in the presence of 100 U/mL SOD. In each incubation, 10 mM pyruvate, 5 mM malate and 1 mM ADP were included. EPR intensities were normalized for the protein content and are shown in panel (b) as mean ± S.E.M. $^{*}p<0.05$, young *vs.* old, by ANOVA and S–N–K *post hoc* analysis; $^{**}p<0.05$, old *vs.* old-C_3, by ANOVA and S–N–K *post hoc* analysis.

[$F(2,64)=7.41$, $p=0.001$] and pairwise comparisons indicated that the old mice had significantly longer average path lengths across blocks of trials compared to both the young ($p<0.0005$) and old C_3-treated groups ($p=0.010$). Additional analyses showed that these effects were mostly due to differences during the third block of trials. However, the performance of the old mice improved dramatically between the third and fourth blocks of trials to the degree that all three groups were performing equivalently by the last block of trials. An ANOVA of the swimming speed data (not shown) yielded a significant main effect of Group for path length [$F(2,64)=12.85$, $p<0.0005$], and subsequent pairwise comparisons showed that the old and old C_3-treated groups swam significantly slower than the young group, ($p<0.0005$), *i.e.* younger mice were ~25% faster than either old group. Thus, latency was not an appropriate measure to evaluate performance on the water navigation task and we used path length instead which would not be affected by differences in swimming speeds. The results from the cued trials suggest that the old mice initially demonstrated impairment on the cued trials possibly from non-associative influences. However, the old mice improved their performance to the degree that they

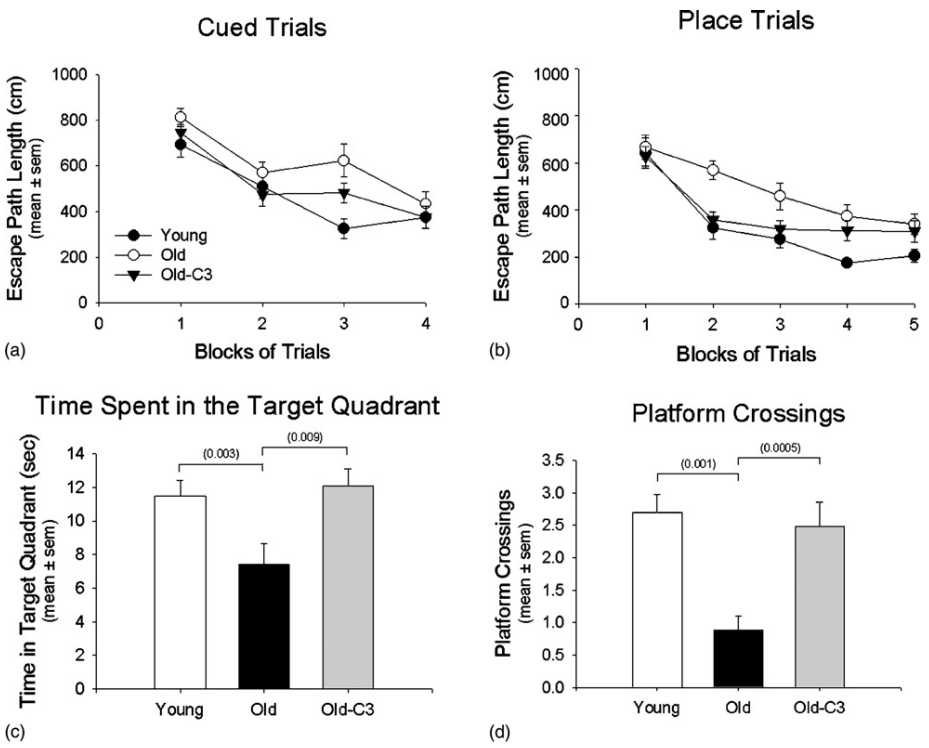

Fig. 5. Chronic SOD mimetic therapy reduces age-dependent decline in spatial learning and memory as indicated by the Morris water maze. Young (6 months, $n = 23$), old control (23–26 months, $n = 18$) and old C_3-treated (23–26 months, $n = 23$) mice were tested on components of the Morris water maze. (a) This graph shows the path length data from the cued (visible platform) trials. An ANOVA and subsequent pairwise comparisons of these data showed that, on the average, the young and old C_3-treated mice had significantly shorter path lengths to the visible platform compared to the old group ($p < 0.0005$ and 0.01, respectively). However, this was mostly due to differences observed during block 3 ($p = 0.001$) which was the only block of trials where the groups differed significantly. Although these results suggest that non-associative influences (*e.g.* sensorimotor or visual disturbances, or alteration in motivation) may have differentially affected the performance of the old mice, it should be noted that they performed as well as the other two groups by the end of training. (b) Performance during the third (platform position) week of spatial trials in terms of path length is depicted. An ANOVA of these data yielded a significant main effect of Group ($p = 0.003$) and a significant Group by Blocks of Trials interaction ($p = 0.032$) suggesting that the groups performed differently across training. Pairwise comparisons showed that the average path length of the young mice was significantly shorter than that of the old group ($p < 0.0005$) with significant differences occurring during blocks 2, 3 and 4 ($p < 0.004$). The average path length of the old C_3-treated mice was also substantially shorter than that of old group ($p = 0.032$) but was not significant according to Bonferroni corrected levels ($p = 0.017$). (c) Probe trial performance concerning retention of the general location of the platform is shown by depicting time spent in the target quadrant where the submerged platform had been located. The groups differed significantly in terms of the time spent in the target quadrant for the probe trial conducted 24-h after the last spatial trial ($p = 0.006$). The young and old C_3-treated groups spent significantly more time in the target quadrant compared to the old mice ($p = 0.009$ and 0.003, respectively) during the 24-h spatial-probe interval. (d) This graph shows retention performance concerning the exact location of the submerged platform by depicting the number of times the mice crossed over the location (*i.e.* number of platform crossings) during the probe trials. When this measure was used, the groups were found to differ significantly in their performance during the 24-h spatial-probe trial intervals. The young and old C_3-treated groups exhibited significantly more platform crossings than the old mice for the 24-h ($p < 0.005$ and 0.001, respectively).

were performing as well as the other two groups by the last block of cued trials, thus demonstrating control-like levels of performance at the end of training. Age- and treatment-related effects on spatial learning evolved with time and the changing of submerged platform locations. Specifically, no differences between groups were observed with regard to escape path length during the first week of spatial trials nor were any differences found between groups on the probe trial variables during the first week (not shown). However, significant differences in group performances were found for the second and third platform locations (weeks 2 and 3). Specifically, a significant main effect of Group was found for week 2 [$F(2,63) = 10.60$, $p < 0.0005$] (data not shown), and subsequent pairwise comparisons showed that the old mice had significantly longer path lengths to the submerged platform compared to the young ($p < 0.0005$) and old C_3-treated groups ($p = 0.014$). The old C_3-treated group had longer path lengths than the young mice ($p = 0.024$) but differences were not significant according to Bonferroni corrected levels (*i.e.* $p = 0.017$). An ANOVA on the place trials data from week 3 (Fig. 5b) also yielded a significant main effect of Group [$F(2,61) = 6.38$, $p = 0.003$], as well as a significant Group by Blocks of Trials interaction [$F(8,244) = 6.38$, $p = 0.046$]. Subsequent comparisons showed that the old group of mice had significantly longer average path lengths than the young mice ($p = 0.001$). The old group also had longer average path lengths than the old C_3-treated mice ($p = 0.032$) but "p values" were not less than Bonferroni corrected levels. Parallel differ-

ences between groups were also found during the probe trials from weeks 2 and 3. That is, an ANOVA on the time spent in the target quadrant data for both retention intervals from week 2 (data not shown) revealed a significant main effect of Group [$F(2,63) = 3.87$, $p = 0.026$], but subsequent pairwise comparisons showed that significant impairment was found only in the old mice relative to the young group at the 24-h retention interval. In contrast, much more robust differences were found for platform crossings, a variable which assesses a more precise memory of the platform location. An ANOVA of these data resulted in a significant main effect of Group [$F(2,63) = 10.33$, $p < 0.0005$] and pairwise comparisons showed that the old mice had significantly fewer platform crossings compared to the young group at 24-h ($p < 0.004$) and 1-h ($p < 0.0005$) intervals and compared to the old C_3-treated group for both intervals ($p < 0.012$ and 0.008, respectively), as well. Similar results were found for the probe trials conducted during week 3 where a significant main effect of Group was found for the time spent in the target quadrant [$F(2,61) = 3.40$, $p = 0.040$]. Subsequent comparisons showed that significant group differences were found only during the 24-h retention probe trial (Fig. 5c) where the old mice spent significantly less time in the target quadrant compared to the young ($p = 0.009$), and to the old C_3-treated groups ($p = 0.003$). Robust differences were found again with regard to platform crossings (Fig. 5d) during the week 3 probe trial where an ANOVA produced a significant main effect of Group [$F(2,61) = 14.31$, $p < 0.0005$]. Again, pairwise comparisons showed that the old mice had significantly fewer platform crossings compared to the young group at 24 and 1-h ($p < 0.0005$) retention intervals and compared to the old C_3-treated group at both intervals ($p < 0.001$ and 0.009, respectively). In summary, we have presented evidence that C_3-treatment helps protect mice against age-related decrements in spatial learning and memory performance. For example, with regard to acquisition performance during the spatial condition, the old C_3-treated mice performed significantly better than the old group in terms of path length during week 2, while effects were marginally non-significant between the two groups during week 3. More robust effects eventually emerged with regard to retention capabilities measured during probe trials, particularly when memory demands were greatest. For example, results from the probe trials conducted during week 3 showed that the C_3-treated mice performed significantly better than the old group and as well as the young mice with regard to platform crossings for both the 24 and 1-h retention intervals and with regard to time spent in the target quadrant for the 24-h retention interval.

4. Discussion

Here we show that an orally-administered SOD mimetic can significantly increase lifespan and reduce mitochondrial free radical production. To our knowledge, this is the first report that a non-transgenic approach other than calorie restriction, a highly complex intervention, can increase the lifespan of a mammal. Non-transgenic C57BL6 mice which received C_3 from 12 months of age had an increase in mean (11%) and median (15%) lifespan (Fig. 2a). Calorie restriction (CR) initiated at 12 months age extends mean lifespan by 14% (median lifespan by 16%) [36], a shift in survival that is similar to ours. Since CR has also been reported to improve free radical handling, this may indicate that there are overlapping mechanisms between these two interventions. If this is true, then one would predict that initiating treatment with C_3 at 2–3 months of age would result in an even greater increase in lifespan, since CR started at 2 months of age increases lifespan by more than 25% [43].

In reviewing the literature on aging and lifespan in standard C57BL6 mice, we found that median lifespans generally reported ranged from 23.5–25 months (females) and 25–27 months (males) across multiple studies [12,15,45]. Our median lifespan was within this range. We also compared our mean lifespan to published values, and found that our data are quite similar to that reported by a number of benchmark studies, including the most recent study from the Biomarkers of Aging Project [43]. That study reported a mean survival for a mixed gender cohort of 775 days (calculated from data therein), with an asymptotic 95% confidence interval from 728 to 822 days when a standard error of 24 days (as estimated by [43]) is used. The mean survival for our uncensored data was 768 days, and for our censored data was 763 days, both of which are within a few days of the Turturro mean, and are well within the 95% confidence interval and are not statistically different from their data. Of note, that study revised downward their previously-published mean survival. In their previous publications [6,44], up to 15% of mice with dermatitis were excluded from survival analysis, but in the 2002 study, all mice were included. In our study, no mice were excluded.

In addition to lifespan extension, C_3 reduced oxidative stress in brain, and superoxide production by isolated mitochondria from aging brain. To evaluate brain oxidative stress *in vivo*, we utilized DHE oxidation as an indicator of cellular superoxide levels. These data showed elevated oxidative stress in all brain regions examined in aged mice in comparison to younger mice, and therefore supports the large body of evidence suggesting that oxidative stress is increased with age in a variety of tissues [40]. Our data also indicate that C_3 treatment was able to attenuate this age-associated increase in oxidative stress and link reduced oxidative stress with enhanced longevity. To determine if C_3 could specifically reduce mitochondrially-produced superoxide, we employed EPR spectroscopy (EPRS) to examine baseline superoxide production of isolated brain mitochondria. The EPRS results showed that there was a significant age-associated increase in brain mitochondrial superoxide production and that C_3 treatment could reverse this increase. We were unable to find any other studies which directly demonstrated baseline superoxide production from isolated mitochondria (that is, without the use of electron transport chain inhibitors) in aging models,

and believe our data are strong evidence that mitochondrial free radical flux is in fact increased with age, at least in brain.

To determine if C_3 was able to alter other biomarkers of aging, we investigated the impact of C_3 treatment on a number of parameters known to change with age, including cognitive function and immune system status. Cognitive decline and impaired sensorimotor performance have both been documented to occur in old mice, although the magnitude and specific types of impairment have been variable across studies [3,33]. We carried out behavioral analyses to determine the effect of age and C_3 treatment on cognitive function of C57BL6 mice using a modified Morris water maze testing protocol that has been shown to be sensitive for detecting episodic-like memory declines in spatial learning and memory in mice resulting from aging and amyloid-β peptide accumulation [16]. As reported previously (36, 37), here we also found a moderate significant age-related decline in performance, particularly in retention capabilities evaluated during probe trials which are reversed by C_3 treatment. It has been shown that levels of oxidative stress may participate in age-associated loss of cognitive function. In addition, age-related impairments in long-term potentiation (LTP) are improved by overexpression of cytosolic SOD-1 [21] and extracellular SOD (EC-SOD) [19] independently. Moreover, age-associated loss of spatial memory as indicated by Morris water maze is prevented by overexpression of EC-SOD [19]. In C57BL/6N Sim mice, fear-conditioning experiments revealed that a decrease in learning and memory can be observed between 8 and 11 months and that chronic treatment with an SOD/catalase mimetic prevents this age-associated loss [30]. Our data are in agreement with these previously published studies which suggest that chronic antioxidant therapy is beneficial for maintaining LTP during aging. In contrast, superoxide was shown to be critical for LTP by the demonstration that cell-permeable SOD mimetics fully block LTP [25] but that cell-impermeable SOD mimetics attenuate but do not completely block LTP through their extracellular activity [26]. While SOD mimetic administration may be beneficial in aging animals, earlier therapy may inhibit LTP as observed in transgenic mice overexpressing human SOD1 examined at 2 months of age [21]. The initiation of C_3-therapy at mid-life may prove to be an important factor for both increasing lifespan and providing a beneficial effect to age-associated deficits in cognition. In addition, the mechanism by which C_3 impacts age-related loss of spatial learning and memory may be through its SOD activity in the extracellular matrix which has been shown to be protective previously [19].

As a robust anti-aging therapy which reduces levels of oxidative stress, CR has produced conflicting results in its ability to slow the loss of age-associated spatial learning [3]. This may be due to lack of (or subtle) effects of CR on the aging nervous system *per se*, the fact that animals which perform poorly on initial testing were often excluded from subsequent testing (*e.g.* [3,33]), potentially biasing the results or the effects of low plasma glucose in CR animals on cognitive function. This latter idea is supported by a study on aged CR rats, which performed worse in the Morris water maze than *ad libitum* fed controls initially, but had a significant improvement in performance when an injection of glucose was given prior to testing [48]. We measured plasma glucose in aged mice, and found no difference between old and old C_3-treated mice (data not shown).

Oxidative stress is known to impair immunological function, and functional decline in the immune system has been well characterized to occur during aging. C_3 treatment reversed aging-associated alterations in the T-lymphocyte $CD4^+/CD8^+$ ratio and reversed age-related loss of B-cells as a percentage of total splenocytes (Supplemental Figure S1).

Taken together, these data suggest that C_3 decreases age-associated mitochondrial superoxide production and improves mitochondrial function, providing support for the mitochondrial theory of aging as one component of the aging process. However, as discussed in detail by Beckman and Ames [2], changes in mitochondrial physiology may reflect more indirect processes (for example, changes in mitochondrial gene expression due to hormone status, inflammation, diet, activity) and may interact with other pathways linked to aging, such as insulin-dependent signaling or the sirtuins. Because C_3 has previously been shown to localize within mitochondria, to enhance survival of $Sod2^{-/-}$ mice [1] and, as shown here, to regulate mitochondrial superoxide production by EPR and extend the lifespan of mice, we think it is likely that some of the actions of C_3 in the current study are directly on mitochondria. However, to what degree and how mitochondria contribute to aging remain important questions and are areas of future study that may provide valuable insights into the complex multifactorial process of aging.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2006.09.014.

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