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Vitamin E at high doses improves survival, neurological performance, and brain mitochondrial function in aging male mice

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Navarro, Ana, Carmen Gómez, María-Jesús Sánchez-Pino, Hipólito González, Manuel J. Bández, Alejandro D. Boveris, and Alberto Boveris. Vitamin E at high doses improves survival, neurological performance, and brain mitochondrial function in aging male mice. *Am J Physiol Regul Integr Comp Physiol* 289: R1392–R1399, 2005. First published July 14, 2005; doi:10.1152/ajpregu.00834.2004.—Male mice receiving vitamin E (5.0 g α-tocopherol acetate/kg of food) from 28 wk of age showed a 40% increased median life span, from 61 ± 4 wk to 85 ± 4 wk, and 17% increased maximal life span, whereas female mice equally supplemented exhibited only 14% increased median life span. The α-tocopherol content of brain and liver was 2.5-times and 7-times increased in male mice, respectively. Vitamin E-supplemented male mice showed a better performance in the tightrope (neuromuscular function) and the T-maze (exploratory activity) tests with improvements of 9–24% at 52 wk and of 28–45% at 78 wk. The rates of electron transfer in brain mitochondria, determined as state 3 oxygen uptake and as NADH-cytochrome *c* reductase and cytochrome oxidase activities, were 16–25% and 35–38% diminished at 52–78 wk. These losses of mitochondrial function were ameliorated by vitamin E supplementation by 37–56% and by 60–66% at the two time points considered. The activities of mitochondrial nitric oxide synthase and Mn-SOD decreased 28–67% upon aging and these effects were partially (41–68%) prevented by vitamin E treatment. Liver mitochondrial activities showed similar effects of aging and of vitamin E supplementation, although less marked. Brain mitochondrial enzymatic activities correlated negatively with the mitochondrial content of protein and lipid oxidation products ($r^2 = 0.58–0.99$, $P < 0.01$), and the rates of respiration and of complex I and IV activities correlated positively ($r^2 = 0.74–0.80$, $P < 0.01$) with success in the behavioral tests and with maximal life span.

mitochondrial nitric oxide synthase; mitochondrial respiration; complex I; complex IV; oxidative damage

THE FREE RADICAL THEORY of aging emerged from the views of Gerschman (1954) (18) who postulated that oxygen free radicals are the common molecular mechanism of oxygen and radiation toxicity, and of Harman (1956) (24) who considered that free radicals, generated as byproducts of biological oxidations, produce random and cumulative cellular damage, which leads to tissue and organ aging. The association between aging and mitochondria is based on the role of the organelles in furnishing cell energy and on the physiological decline of organ energy expenditure and physiological function associated with aging. The mitochondrial hypothesis of aging (4, 25, 44) focuses on mitochondria as the pacemakers of tissue aging due to the continuous production of reactive oxygen and nitrogen species in these organelles. Two initial free radicals, $O_2^{\cdot -}$ (8, 11) and nitric oxide (NO; 13, 19, 21), are continuously produced in mitochondria, and other reactive species such as H_2O_2, $ONOO^-$, $HO^\cdot$, $ROO^\cdot$, and $^1O_2$ are derived from the primary production of $O_2^{\cdot -}$ and NO (8). An increase in the steady state concentration of any of the reactive oxygen or nitrogen species and the self-propagation of free radical reactions constitute the chemical basis of an increased oxidative damage to lipid and proteins, a situation that when sustained constitutes a factor that promotes a faster aging (44).

The role of α-tocopherol as a chain-breaking antioxidant is well characterized in vitro; moreover, it is considered the major lipophilic antioxidant in the human body, specifically by its reaction with $ROO^\cdot$ radicals (27, 43). Thus vitamin E has been extensively assayed in experimental animal diseases and in the protection and treatment of human diseases. It has been claimed that vitamin E supplementation prevents or ameliorates chronic and age-associated diseases such as cardiovascular disease, chronic inflammation, and neurological disorders (2, 14). Recently, α-tocopherol has been recognized to have also important nonantioxidant effects in the regulations of cell signaling and gene expression (3, 48). However, there is no consistent information concerning vitamin E effects on the median and maximal life span of experimental animals and on the decline of physiological functions associated with aging. Morley and Trainor (33) reported that vitamin E at 400 mg/kg throughout mice life had no effect on median life span (about 116 wk), whereas Blackett and Hall (6) using 2,500 mg/kg reported an increase in rat median life span but not in maximal life span. On the other hand, Reckelhoff et al. (41) giving 5,000 mg/kg from 52 to 88 wk of rat age observed a prevention in the decline of renal function upon aging. In this paper, we report the beneficial effects of a high doses of α-tocopherol acetate, 5.0 g/kg of food, a level similar to the one used by Reckelhoff et al. (41), on *1*) mice survival (median and maximal life span), *2*) neuromuscular function and exploratory activities, *3*) content of lipid and protein oxidation products in brain and liver mitochondria, *4*) mitochondrial respiration and oxidative phosphorylation, and *5*) enzyme activities in brain and liver mitochondria. The rationale of using the two organs was to consider a postmitotic organ with low mitochondrial turnover, the brain, and a mitotic tissue with a higher mitochondrial turnover, the liver (35).

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MATERIALS AND METHODS

Animals and vitamin E supplementation. Mice of the CD-1/UCadiz strain inbred at the Department of Experimental Animals of the University of Cadiz (36, 37) were housed in groups of 5 mice at 24 ± 1°C with 12:12-h light-dark cycles and had full access to water and food. The control group received a standard laboratory animal food (A04 diet, Panlab LS, Barcelona, Spain) with 29 ± 1 mg α-tocopherol/kg of food, whereas vitamin E-supplemented mice received the same food supplemented with 5.0 g *dl*-RRR-α-tocopherol acetate/kg of food (analyzed 4.5 ± 0.1 g α-tocopherol/kg) from 28 wk of age for their entire lives. Vitamin E supplementation was started in young adult mice to avoid effects during development and normal growth. Mice were weekly weighed and periodically checked to verify their pathogen-free condition. Animal experiments were carried out in accordance with current regulations such as the 86/609/CEE European Community and the "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society.

Survival curves. Male mice ($n = 50$), vitamin E-supplemented male mice ($n = 40$), female mice ($n = 50$), and vitamin E-supplemented female mice ($n = 40$) were used to construct the survival curves and the statistics for equality of survival distributions. Survival was daily controlled.

Behavioral tests. Individual mice were subjected every 2 wk to two behavioral assays, the tightrope test (31, 36, 37) and the T-shaped maze test (36, 37). In the tightrope test to evaluate the neuromuscular coordination, mice were placed hanging from their hind legs in the middle of a 60-cm tightrope, and the test was considered successful when mice reached the column at the end of the rope in less of 30 s. In the test to evaluate the spontaneous exploratory and cognitive activities, mice were challenged in a T-shaped maze of 50-cm arms; the test was considered successful when mice moved toward the T-intersection in less than 30 s.

Determination of α-tocopherol. Tissue homogenates were added with 1/50 of 50 mM butylated hydroxytoluene and extracted with 4 ml hexane/ml of homogenate. Mouse food was extracted 1/10 (wt/vol) with hexane. The mixtures were centrifuged at 1,000 *g* for 2 min to separate a clean organic layer. A hexane aliquot was dried under N_2, and the residue dissolved in 0.5–2.0 ml of ethanol and filtered through a 0.22-μm pore membrane. α-Tocopherol was determined by HPLC using a Bioanalytical System electrochemical detector (LC-4C) at 0.6-V coupled to an isocratic delivery system with the samples injected through a Reodhyne system to a 4-μm-Nova-Pak column of 4 × 150 mm. Separation was performed with the mobile phase, 98% methanol, at a flow rate of 1 ml/min (28).

Isolation of mitochondria. Brain and liver mitochondria were isolated from the organs homogenized in 0.23 M mannitol, 0.07 M sucrose, 15 mM MOPS-KOH (pH 7.2) at a ratio of 1 g of tissue/9 ml of homogenization medium in a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 700 *g* for 10 min, and the supernatant centrifuged at 8,000 *g* for 10 min to precipitate mitochondria that were washed in the same conditions. Mitochondrial proteins, assayed by the Folin reagent, were adjusted to about 20 mg/ml, and the samples were immediately used for respiratory measurements or frozen in liquid N_2 and kept at −80°C. Mitochondria were disrupted and homogenized by twice freezing and thawing and by passage through 15/10 tuberculin needles and called mitochondrial membranes (37).

Mitochondrial oxygen consumption. Oxygen uptake was determined with a Clark electrode in a 1.5-ml chamber at 30°C, in an air-saturated reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris·HCl, pH 7.4, 1 mM EDTA, 5 mM phosphate, 4 mM $MgCl_2$, and 0.5–0.7 mg mitochondrial protein/ml, at pH 7.4. Respiratory rates were determined with either 6 mM malate and 6 mM glutamate or 10 mM succinate as substrate, and state 3 active respiration was established by addition of 0.5 mM ADP. Oxygen uptake is expressed as nanograms of oxygen per minute per milligram protein membrane (9).

Biochemical markers of oxidative stress. The mitochondrial content of thiobarbituric acid-reactive substances (TBARS) and of protein carbonyls were determined in mitochondrial membranes as originally described by Fraga et al. (17) and by Oliver et al. (38), respectively, and modified as described by Navarro et al. (37). For TBARS determination, 1 ml of mitochondrial membranes was added with 2 ml of 0.1 N HCl, 0.3 ml of phosphotungstic acid, and 1 ml of 0.67% 2-thiobarbituric acid, heated 30 min in boiling water, and extracted with 5 ml 1-butanol. The absorption of the butanol phase, separated by a brief centrifugation, was measured at 535 nm ($\epsilon = 153\ mM^{-1}\ cm^{-1}$) and expressed as picomoles TBARS/milligram of mitochondrial protein. For protein carbonyl determination, 0.05 ml of mitochondrial membranes was supplemented with 0.05 ml of 10% trichloroacetic acid (TCA); the precipitated proteins were suspended in 0.05 ml of 0.2% 2,4-dinitrophenyl hydrazine, incubated 1 h at 37°C, precipitated again with TCA, centrifuged, washed with ethanol:ethyl acetate (50:50), dissolved in 6 mM guanidine hydrochloride in phosphate buffer (pH 6.5), and the absorbance was determined at 370 nm ($\epsilon = 21\ mM^{-1}\ cm^{-1}$). Protein carbonyls are expressed as picomoles per milligram of mitochondrial protein.

Mitochondrial electron transfer activities. The activities of complexes I-III, II-III, and IV were determined spectrophotometrically at 30°C with mitochondrial membranes suspended in 100 mM phosphate buffer (pH 7.4) with the corresponding substrates (36, 37). For NADH-cytochrome *c* (complexes I-III) and succinate-cytochrome *c* reductase (complexes II-III) activities, mitochondrial membranes were supplemented with 0.2 mM NADH or with 20 mM succinate as substrates, 0.1 mM cytochrome c^{3+}, and 1 mM KCN, and the enzymatic activity was determined at 550 nm ($\epsilon = 19\ mM^{-1}\ cm^{-1}$) and expressed as nanomoles cytochrome *c* reduced/mg protein. Cytochrome oxidase (complex IV) activity was determined in the same phosphate buffer added with 0.1 mM cytochrome c^{2+}. The rate of cytochrome *c* oxidation was calculated as first-order reaction constant k' per milligram protein and expressed as nanomoles cytochrome *c* oxidized at 10 μM cytochrome *c*/mg protein.

Spectrophotometric determination of mtNOS (mitochondrial nitric oxide synthase) activity. Mitochondrial NO production was determined by the oxyhemoglobin (HbO_2) oxidation assay as described (7). The reaction medium consisted of 0.1 mM NADPH, 0.2 mM arginine, 1 mM $CaCl_2$, 4 μM Cu, Zn-SOD, 0.1 μM catalase, and 25 μM HbO_2 heme, in 50 mM phosphate buffer at pH 7.2 (liver) and at pH 5.8 (brain). A diode array spectrophotometer (model 8453 Agilent, Palo Alto, CA) was used to follow the absorbance change at 577 nm with a reference wavelength at the isosbestic point of 591 nm ($\epsilon_{577-591} = 11.2$ mM/cm). Production of NO was calculated from the absorbance change that was inhibited by 2 mM N^G-monomethyl-L-arginine, usually 88–96%, and expressed in nanomoles NO per minute per milligram protein.

Mitochondrial superoxide dismutase activity. Dismutase activity was determined by the adrenochrome spectrophotometric assay followed at 480 nm ($\epsilon = 4.0\ mM^{-1}\ cm^{-1}$) in a reaction medium containing 1 mM epinephrine, 1 mM KCN, and 50 mM glycine/KOH (pH 10.0). One Misra-Fridovich unit of enzyme activity, 50% inhibition of the rate of spontaneous adrenochrome formation, is equivalent to 11 pmol of Mn-SOD active center (22, 32).

Statistics. The survival curves were analyzed by the Kaplan-Meier test. The numbers in tables and figures are mean values ± SE. Differences between groups were analyzed by the Student-Newman-Keuls post hoc test after significant one-way ANOVA. A P value of <0.05 was considered statistically significant. Statistical analyses were carried out using a statistical package (SPSS 11.5 for Windows).

RESULTS

Mice supplemented with vitamin E from 28 wk of age showed increased survival, more marked in males than in females. Male mice receiving vitamin E exhibited a marked (40%) increase in median life span and a moderate (17%) increase in maximal life span (Fig. 1*A*). Female mice, receiving the same vitamin E supplementation showed a smaller vitamin E effect, with a 14% increased median life span and no effect on maximal life span (Fig. 1*B*). The longer life span of females is in agreement with the lower mitochondrial production of oxidants in females than in males and the downregulation of oxidant production by estrogenic hormones (47). There were no weight differences between vitamin E-supplemented and control male or female mice. The next steps of this study, neurological performance and biochemical assays, were conducted only in male mice, considering the lower vitamin E effect in female mice.

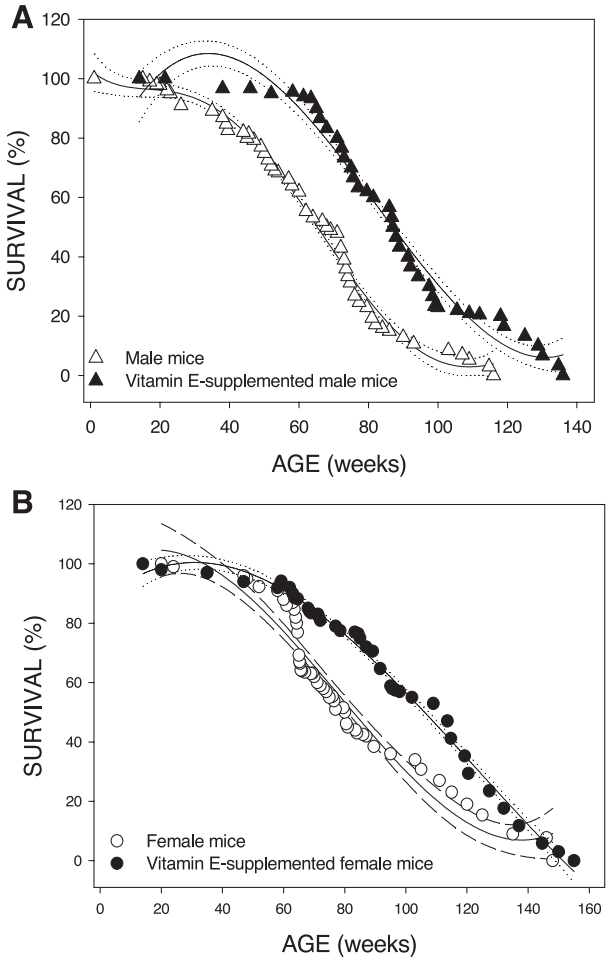

Fig. 1. *A*: mice survival curves. Male mice ($n = 50$): median life span, 61 ± 4 wk; maximal life span, 116 ± 4 wk; vitamin E-supplemented male mice ($n = 40$): median life span, 85 ± 4 wk; maximal life span, 136 ± 4 wk. Statistics for equality of survival distributions for male: log rank: 15.7, $P < 0.0001$; Breslow: 15.1, $P < 0.0001$; Tarone-Ware: 15.8, $P < 0.0001$. *B*: female mice ($n = 50$): median life span, 78 ± 4 wk; maximal life span, 148 ± 4 wk; vitamin E-supplemented female mice ($n = 40$): median life span, 88 ± 5 wk; maximal life span, 155 ± 4 wk. Statistics for equality of survival distributions for female: log rank: 3.4, not significant (NS); Breslow: 2.6, NS; Tarone-Ware: 3.2, NS.

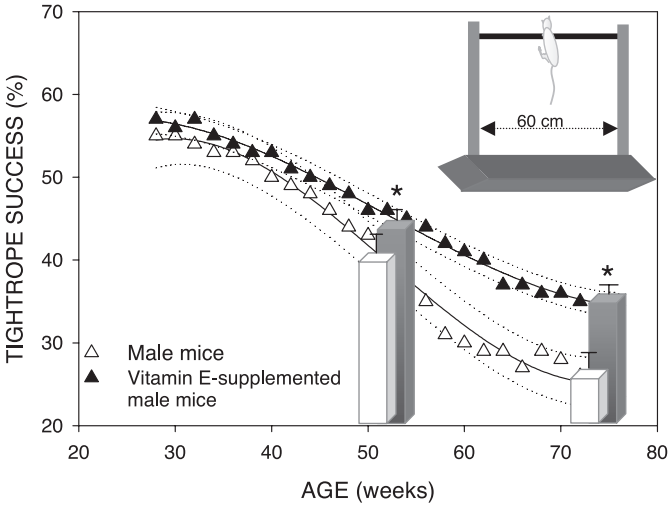

Fig. 2. Effect of vitamin E supplementation on male mice neuromuscular function. The number of animals tested at 28, 52, and 76 wk of age were 140, 102, and 70 mice, respectively. The bars at 52 and 76 wk illustrate the values at the two time points. The 95% confidence interval is indicated by the dotted lines. Control mice: $r^2 = 0.97$; vitamin E-supplemented mice: $r^2 = 0.99$. *P* value for paired *t*-test < 0.00001. $*P < 0.05$.

Mice were individually tested for neurological performance every 2 wk, starting at 28 wk of age and for their entire lives. Neuromuscular coordination was assessed with the tightrope test and exploratory function was tested in a T-shaped maze (31, 36, 37). Success in both tests decreased continuously upon mice aging (Figs. 2 and 3). Success in the tightrope test, considering as reference the performance of control (young) mice at 28 wk of age, was decreased by 25% at 52 wk (adult mice) and by 52% at 76 wk (senescent mice). This loss in motor coordination was partially ameliorated, by 28–29% at 52 and 76 wk in vitamin E-supplemented mice (Fig. 2). Similarly, success in the T-maze, taking as reference the performance of mice at 28 wk of age, decreased by 29% at 52 wk and by 41% at 76 wk, and the loss in exploratory activity was clearly ameliorated, by 59% and 39% at 52 and 76 wk, respectively, by vitamin E supplementation (Fig. 3).

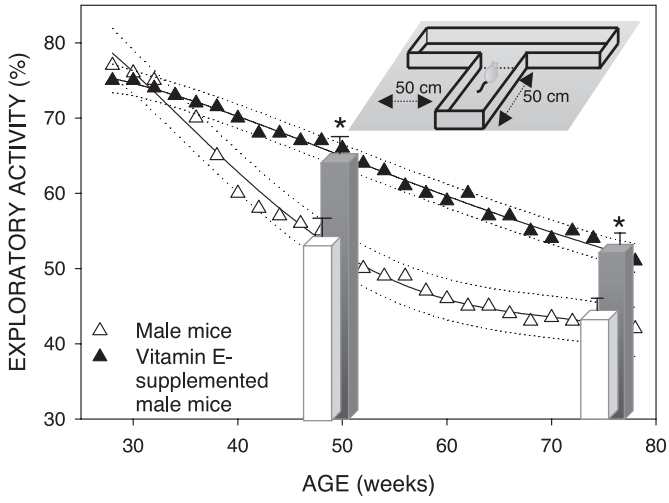

Fig. 3. Effect of vitamin E supplementation on male mice exploratory function. Mice tested and confidence interval as in Fig. 2. Control mice: $r^2 = 0.99$; vitamin E-supplemented mice: $r^2 = 0.99$. *P* value for paired *t*-test < 0.00001. $*P < 0.05$.

Table 1. *Vitamin E content in the brain and liver of mice fed diets with standard and high levels of vitamin E*

Organ/Group/Marker	28 wk	52 wk	76 wk
Brain			
Control	11.5±0.7	11.0±0.7	10.1±0.8
Vitamin E-supplemented		27.8±1.5*	26.2±1.4*
Liver			
Control	27±2	26±2	23±3
Vitamin E-supplemented		196±11*	168±12*

Values are presented as means ± SE and are given in nanomoles per gram tissue; eight mice in each group. *$P < 0.05$ for vitamin E supplementation. ANOVA parameters: brain, $F_{(4,39)} = 66$, $P < 0.0001$; and liver, $F_{(4,39)} = 141$, $P < 0.0001$.

The content of α-tocopherol in brain and liver was markedly augmented, 2.5 times (brain) and 7 times (liver) after vitamin E supplementation (Table 1); effects that agree with previous reports (26, 28).

The mitochondrial content of lipid and protein oxidation products, an indication of free radical-mediated reactions and oxidative damage, was increased in the brain and liver of aging mice, and the effect was partially prevented by vitamin E (Table 2). The protein carbonyl content of brain mitochondria, taking 28-wk-mice as reference, increased by 33% and 69% at 52 and 76 wk of age, and this increase was markedly prevented, 76% and 65%, by vitamin E supplementation at the two respective time points. Similarly, the brain mitochondrial content of TBARS increased by 40% and 60% at wk 52 and 76, and the effect was prevented by 35–37% at the two time points by vitamin E supplementation. Protein carbonyl and TBARS contents in liver mitochondria followed the same pattern in control and vitamin E-supplemented mice, but the changes were less marked (Table 2), in agreement with the higher age-associated oxidative damage observed in brain compared with liver (35–37).

Mitochondrial enzymatic activities, NADH-cytochrome *c* reductase, cytochrome oxidase, mitochondrial nitric oxide synthase (mtNOS) and Mn-SOD, decreased almost linearly upon mice aging from 28 to 76 wk, in the range of 35–67% in brain and of 37–40% in liver (Table 3). A decreased NADH-cytochrome *c* reductase activity with an unaffected succinate-cytochrome *c* reductase activity correspond to a decreased complex I (NADH-ubiquinone reductase) activity (35–37). Vitamin E supplementation prevented these age-associated decreases in enzymatic activity by 42–66% in brain and by 16–50% in liver. Moreover, vitamin E supplementation was able to prevent the decrease in the activities of brain enzymes that are mitochondrial markers of aging (34): mtNOS (by 95%), Mn-SOD (by 60%), and NADH-cytochrome *c* reductase and cytochrome oxidase activities (by 35–36%).

The effects of aging and vitamin E on mitochondrial O_2 uptake and oxidative phosphorylation were determined in mice brain and liver after 24 wk of vitamin E supplementation. The rates of respiration of brain mitochondria in active state 3 with malate-glutamate and succinate as substrates were 21–24%

Table 2. *Effect of vitamin E on protein and lipid oxidation products in brain and liver mitochondria of aging mice*

Organ/Group/Marker	28 wk	52 wk	76 wk
Brain			
Protein carbonyls			
Control	51±4	68±4*	89±5*
Vitamin E-supplemented		55±5	63±5†
Tbars			
Control	5.3±0.4	7.4±0.4*	8.6±0.4*
Vitamin E-supplemented		6.7±0.4	7.1±0.4†
Liver			
Protein carbonyls			
Control	120±6	145±6*	178±6*
Vitamin E-supplemented		141±6	157±6†
TBARS			
Control	3.8±0.3	4.6±0.4	5.6±0.4*
Vitamin E-supplemented		4.0±0.4	4.2±0.4†

Values are presented as means ± SE and are given in picomoles per milligram mitochondrial protein; 12 mice in each group. *$P < 0.05$ for aging, compared with 28-wk-old mice. †$P < 0.05$ for vitamin E-supplemented compared with control mice. ANOVA parameters: brain, protein carbonyls $F_{(4,59)} = 11$, $P < 0.001$: thiobarbituric acid-reactive substances (TBARS) $F_{(4,59)} = 9.3$, $P < 0.001$; liver, protein carbonyls $F_{(4,59)} = 11.7$, $P < 0.001$; TBARS $F_{(4,59)} = 3.8$, $P < 0.01$.

Table 3. *Effect of vitamin E on enzymatic activities of brain and liver mitochondrial membranes in aging mice*

Organ/Enzyme Activity/ Group	28 wk	52 wk	76 wk
Brain			
NADH-cytochrome *c* reductase			
Control	322±10	270±10*	210±10*
Vitamin E-supplemented		290±10	283±11†
Succinate-cytochrome *c* reductase			
Control	125±9	123±9	130±9
Vitamin E-supplemented		129±9	128±9
Cytochrome oxidase			
Control	120±8	90±8*	75±8*
Vitamin E-supplemented		107±8	102±8†
mtNOS			
Control	0.63±0.05	0.35±0.04*	0.21±0.03*
Vitamin E-supplemented		0.54±0.05†	0.41±0.05†
Mn-SOD			
Control	18±2	13±2	9±2*
Vitamin E-supplemented		15±2	14±2†
Liver			
NADH-cytochrome *c* reductase			
Control	443±14	361±14*	313±14*
Vitamin E-supplemented		370±14	351±14†
Succinate-cytochrome *c* reductase			
Control	161±10	158±10	163±10
Vitamin E-supplemented		162±10	166±10
Cytochrome oxidase			
Control	131±8	99±8	78±8*
Vitamin E-supplemented		108±8	87±9
mtNOS			
Control	0.73±0.06	0.62±0.05	0.44±0.05*
Vitamin E-supplemented		0.65±0.05	0.57±0.05†
Mn-SOD			
Control	27±3	24±2	17±2
Vitamin E-supplemented		25±2	22±2

Values are presented as means ± SE. NADH- and succinate-cytochrome *c* reductase, and cytochrome oxidase activities are expressed in nmol cytochrome *c* (reduced or oxidized) $\cdot min^{\cdot -1}$ mg $protein^{-1}$, mitochondrial nitric oxide synthase (mtNOS) in nmol $NO \cdot min^{-1} \cdot$mg $protein^{-1}$, and Mn-SOD in pmol SOD/mg protein; 12 mice in each group. *$P < 0.05$ for aging, compared with 28-wk-old mice. †$P < 0.05$ for vitamin E-supplemented compared with control mice. ANOVA parameters: brain, NADH-cytochrome *c* reductase $F_{(4,59)} = 15.3$, $P < 0.001$; succinate-cytochrome *c* reductase $F_{(4,59)} = 0.2$, NS; cytochrome oxidase $F_{(4,59)} = 4.1$, $P < 0.01$; mtNOS $F_{(4,59)} = 13$, $P < 0.001$; Mn-SOD $F_{(4,59)} = 3.5$, $P < 0.05$; liver, NADH-cytochrome *c* reductase $F_{(4,59)} = 11.6$, $P < 0.001$; succinate-cytochrome *c* reductase $F_{(4,59)} = 0.1$, NS; cytochrome oxidase $F_{(4,59)} = 6.1$, $P < 0.001$; mtNOS $F_{(4,59)} = 4.1$, $P < 0.01$; Mn-SOD $F_{(4,59)} = 2.5$, NS.

Table 4. *Effect of high doses of vitamin E on the oxidative phosphorylation of mouse brain mitochondria*

Substrate/Determination	Control		Vitamin E-Supplemented
	28 wk	52 wk	52 wk
Malate-glutamate			
State 4 respiration	27±2	28±2	26±3
State 3 respiration	84±6	66±5*	74±6†
Respiratory control	3.1±0.2	2.4±0.2*	2.8±0.2
ADP:O ratio	2.58±0.10	2.52±0.09	2.54±0.10
State 3 + arginine	84±5	63±4*	76±5†
State 3 + L-NAME	104±8	72±7*	92±8†
mtNOS-regulated O_2 uptake	20±3 (19 %)	9±3 (12 %)*	16±4 (17 %)
Succinate			
State 4 respiration	50±5	53±5	54±4
State 3 respiration	182±10	138±9*	160±9†
Respiratory control	3.6±0.2	2.5±0.2*	3.0±0.2†
ADP:O ratio	1.78±0.11	1.75±0.10	1.76±0.10
State 3 + arginine	176±8	134±7*	152±8†
State 3 + L-NAME	216±10	154±8*	181±7†
mtNOS-regulated O_2 uptake	40±5 (22 %)	20±3 (14 %)	29±4 (18 %)

Values are expressed as means ± SE; 9 mice in each group. Oxygen uptake (state 3 and state 4) is expressed as nanograms of oxygen per minute per milligram protein membrane, and respiratory control is the ratio state 3/state 4 respiration. The % indicated in mtNOS-regulated O_2 uptake is the % of state 3 respiration as: [(state 3 + L-NAME) − (state 3 + arginine)]/state 3 respiration] × 100. *$P < 0.05$ for aging, compared with 28-wk-old mice. †$P < 0.05$ for vitamin E-supplemented mice, compared with control mice. ANOVA parameters, for malate-glutamate: state 3, $F_{(2,26)} = 3.3$, $P < 0.05$; state 3 + arginine, $F_{(2,26)} = 5.3$, $P < 0.01$; state 3 + L-NAME, $F_{(2,26)} = 5.4$, $P < 0.01$; and for succinate: state 3, $F_{(2,26)} = 6.2$, $P < 0.005$; state 3 + arginine, $F_{(2,26)} = 8.2$, $P < 0.005$; state 3 + L-NAME, $F_{(2,26)} = 15.1$, $P < 0.0001$.

decreased in 52-wk-old mice, an effect that was 48–50% prevented by vitamin E supplementation (Table 4). Respiratory control and ADP:O ratios were neither affected by aging nor by vitamin E supplementation. The functional activity of mtNOS in the regulation of state 3 O_2 uptake was decreased to 45–50% by aging and the loss was 45–64% prevented by vitamin E, in clear agreement with the effects observed in mtNOS biochemical activity ($r = 0.94$, $P < 0.01$). The effects of aging and vitamin E supplementation on liver mitochondria, considering respiration, oxidative phosphorylation, and mtNOS functional activity, were qualitatively similar to the ones observed in brain mitochondria, but less marked (Table 5).

DISCUSSION

The present study shows the beneficial effects of high doses of vitamin E on the median and maximal life span of male mice, an effect that was parallel to a beneficial effect on the declines of neurological performance and mitochondrial function associated with aging. The marked increase (40%) in median life span, from 61 to 85 wk, and the moderate increase (17%) in maximal life span observed in male mice is properly described as a delay in the onset of the almost linear decay in mice survival, as shown in Fig. 1. The mice strain used in this study, CD-1/UCadiz, belong to a senescence-accelerated strain

Table 5. *Effect of high doses of vitamin E on the oxidative phosphorylation of mouse liver mitochondria*

Substrate/Determination	Control		Vitamin E-Supplemented
	28 wk	52 wk	52 wk
Malate-glutamate			
State 4 respiration	25±2	25±3	24±2
State 3 respiration	102±9	72±6*	80±7
Respiratory control	4.1±0.2	2.9±0.1*	3.3±0.1
ADP:O ratio	2.61±0.10	2.65±0.09	2.62±0.10
State 3 + arginine	70±6	54±5*	62±4†
State 3 + L-NAME	109±9	76±6*	95±8†
mtNOS-regulated O_2 uptake	39±3 (38 %)	22±2*(30 %)	33±3† (41 %)
Succinate			
State 4 respiration	54±4	46±5	53±5
State 3 respiration	179±10	145±8*	177±10†
Respiratory control	3.3±0.2	2.8±0.2*	3.3±0.2†
ADP:O ratio	1.78±0.11	1.75±0.10	1.76±0.10
State 3 + arginine	144±10	113±9*	155±10†
State 3 + L-NAME	194±12	152±10*	203±13†
mtNOS-regulated O_2 uptake	50±4 (28 %)	39±3*(27 %)	48±4† (27 %)

Values are presented as means ± SE; 9 mice in each group. Units and % as indicated in Table 4. *$P < 0.05$ for aging, compared with 28-wk-old mice. †$P < 0.05$ for vitamin E-supplemented mice, compared with control mice. ANOVA parameters: for malate-glutamate: state 3, $F_{(2,26)} = 5.2$, $P < 0.01$; state 3 + arginine, $F_{(2,26)} = 3.7$, $P < 0.05$; state 3 + L-NAME, $F_{(2,26)} = 5.0$, $P < 0.01$; and for succinate: state 3, $F_{(2,26)} = 4.6$, $P < 0.05$; state 3 + arginine, $F_{(2,26)} = 6.1$, $P < 0.005$; state 3 + L-NAME, $F_{(2,26)} = 5.4$, $P < 0.01$.

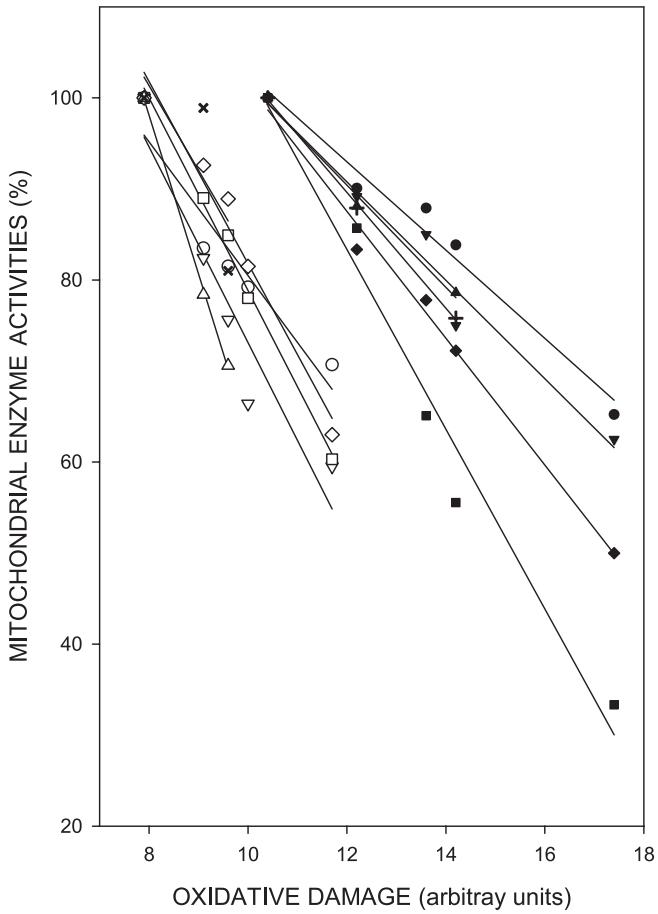

Fig. 4. Correlation between mitochondrial oxidative damage and respiration rates and enzymatic activities in mouse brain and liver mitochondria. Oxidative damage is expressed in arbitrary units [1 AU = TBARS (nmol/mg protein) + 0.1 protein carbonyls (nmol/mg protein)/2]. Enzymatic activities, expressed as % of the activity at 28 wk, are NADH-cytochrome *c* reductase (●, ○); cytochrome oxidase (▼, ▽); mtNOS (■, □); Mn-SOD (◆, ◇), and state 3 respiration rates with malate-glutamate (▲, △) and succinate (+, ×). Brain: solid symbols and +; liver open symbols and ×. Brain: oxidative damage vs. NADH-cytochrome *c* reductase, $r^2 = 0.97$, $P < 0.0001$; brain oxidative damage vs. cytochrome oxidase, $r^2 = 0.97$, $P < 0.0001$; brain oxidative damage vs. mtNOS, $r^2 = 0.97$, $P < 0.01$; brain oxidative damage vs. Mn-SOD, $r^2 = 0.99$, $P < 0.001$; malate-glutamate, $r^2 = 0.99$, $P < 0.001$; succinate, $r^2 = 0.99$, $P < 0.01$. Liver: oxidative damage vs. NADH-cytochrome *c* reductase, $r^2 = 0.91$, $P < 0.0001$; liver oxidative damage vs. cytochrome oxidase, $r^2 = 0.91$, $P < 0.0001$; liver oxidative damage vs. mtNOS, $r^2 = 0.99$, $P < 0.0001$; liver oxidative damage vs. Mn-SOD, $r^2 = 0.97$, $P < 0.0001$; malate-glutamate, $r^2 = 0.99$, $P < 0.01$; succinate, $r^2 = 0.58$, $P < 0.001$.

similar to AKR, SAM, NZB/Lac, and SJL/J, which exhibit a median life span of 36–57 wk and a maximal life span of 52–83 wk (15). In comparing rodent strains, life span is directly related to the quality of neurological and endocrine responses and to the performance in mazes and behavioral tests (12, 16, 20), which seems to reflect the fact that aging is characterized by a general decline of physiological functions with a more marked effect in the ones that depend on central nervous system functions. In a mechanistic approach, the decreases in neuromuscular coordination and maze performance upon aging were found directly related to the brain content of lipid and protein oxidation products (16, 36, 37). In this study, mice supplemented with vitamin E from 28 wk of age exhibited a marked improvement in the neurological performances, determined in the tightrope and the T-maze tests, at the adult (52 wk) and senescent (76 wk) time points (Figs. 2 and 3). Prevention of the age-associated decline in mouse neurological functions has been also observed after supplementation with acetyl-carnitine and lipoic acid (23, 29) and with flavonoid-rich vegetable extracts (5), effects that were interpreted as due to protection or remediation of oxidative damage.

It is worthwhile to make a few comments regarding the current use of high doses of vitamin E in the treatment of disorders of the central nervous system in humans (46). Beneficial effects of vitamin E on cognitive capacity have been reported in Alzheimer's disease patients receiving 2,000 mg vitamin E/day (42), and in healthy elders with 1,200 mg/day (30). However, the latter effects are currently contested (39). In the present study, mice received 5,000 mg α-tocopherol acetate/kg of food, which corresponds to 0.20 mg /kJ of mouse basal metabolic rate, an expression that allows a comparison with the human intake of vitamin E, in spite of the enormous difference in body weight in both species. It is considered, for mice and humans, that Energy expenditure = A × weight (kg) $^{0.67}$, where A = 1.80 m^2 /17.2 kg × 3,600 $kJ \cdot m^{2-1} \cdot day^{-1}$ = 377 kg^{-1} × kJ/day. Basal metabolic rates are 6.48 MJ/day for humans (70 kg) and 52 kJ/day for mice [male, 52 g at 52 wk of age (42), 2.1 g/food/day], data that indicates that the mouse vitamin E intake of this study corresponds to a human daily intake of 1,296 mg vitamin E/day.

A cumulative oxidative damage and a reduction of the mitochondrial capacity to produce ATP in the organs and tissues of aged mammals are the two main concepts of the mitochondrial hypothesis of aging (4, 45, 47).

Indeed, brain and liver mitochondria of aging mice showed an increased content of oxidation products, TBARS and protein carbonyls (Table 2) in agreement with reports of increased TBARS, hydroperoxides, protein carbonyls and 8-HO-guanosine in the tissues of aged mammals (4).

Reduced ATP production can occur through reduction of either mitochondrial mass or the specific rate of ATP synthesis.

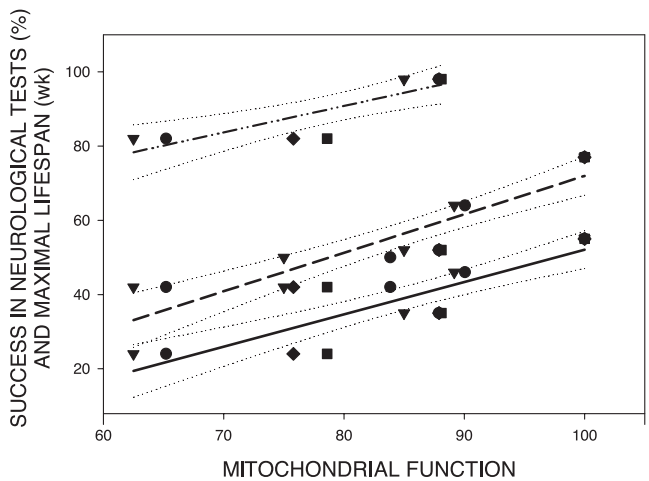

Fig. 5. Correlation between brain mitochondrial electron transfer activities [mean value of the % of state 3 respiration with malate-glutamate (■) and succinate (◆), NADH-cytochrome *c* reductase (●), and cytochrome oxidase (▼) at 28 (taken as 100%)], 52, and 78 wk of age with success in the tightrope, and the T-maze tests at the same age points; and with maximal life span. Correlations of enzyme activities to tightrope success (——), $r^2 = 0.75$, $P < 0.001$; to exploratory activity success (- - - -), $r^2 = 0.80$, $P < 0.001$; and to maximal life span (– .. –), $r^2 = 0.74$, $P < 0.001$.

Recently, we reported that the mitochondrial content of rat brain and liver are not reduced in aging (35). Mitochondrial function encompasses electron transfer in the mitochondrial respiratory chain with H^+ release to the intermembrane space, and H^+ reentry to the matrix through F_0 with ATP synthesis. An age-dependent impairment of mitochondrial function may comprise: *1*) decreased electron transfer rates, *2*) failure in maintaining of the H^+ electrochemical gradient, and *3*) impairment of the H^+-driven ATP synthesis. In this study, we determined the electron transfer rates as the enzymatic activities of mitochondrial membranes and as state 3 O_2 uptake in whole mitochondria (point *1*) and the coupled phosphorylation in mitochondria (points *2* and *3*). The age-associated decline of electron transfer and of state 3 respiration was identified as a selective impairment of complexes I and IV activities in brain and liver (Table 3). The loss in electron transfer and respiration rates was markedly prevented by vitamin E supplementation. Both, complex I and complex IV are then markers of aging (4, 35–37).

The effects of aging and vitamin E supplementation on state 3 respiration (Tables 4 and 5) agree with the effects on electron transfer (Table 3); the rates of mitochondrial O_2 uptake correlated with the complex I and IV activities in brain and liver ($r^2 = 0.98$, $P < 0.01$). Interestingly, aging from 28 to 52 wk did not produce uncoupling or a decrease in the ADP:O ratios in brain and liver mitochondria, indicating that energy conservation and ATP synthesis were unaffected by aging.

The activities of the inner membrane bound mtNOS and of the matrix enzyme MnSOD in brain and liver mitochondria also decreased upon aging, in agreement with earlier reports (4, 36–39, 45) and with the concept of specificity rather than randomness in the inactivation of mitochondrial enzymes. The activity of mtNOS was decreased by 44–66% and Mn-SOD by 28–50% at 52–78 wk of mice age, effects that were markedly prevented by vitamin E supplementation.

Two interesting correlations were observed by analyses focused in the mitochondrial enzyme activities referred in this study. The first one is the inverse relationship between oxidative damage and enzymatic activities in brain and liver (Fig. 4), consistent with earlier reports on brain, liver, kidney, and heart mitochondrial membranes (4, 35–37). The reduced enzyme activities are understood as due to oxidized and damaged proteins, and not to a direct inhibitory effect of lipid oxidation products (i.e., malonaldehyde) due to the high dilution of the enzymes in the assays in which the reduced rates were observed.

The second one is the positive linear correlation exerted between brain mitochondrial state 3 O_2 uptake and complexes I and IV activities, as the independent variable, and the behavioral performances in the tightrope and T-maze test and maximal life span, as the dependent variables (Fig. 5). Applying the concept of the rate-limiting step in complex systems from the most simple to the most complex, it is apparent that decreased electron transfer rates and limited respiration and energy supply are the basis of the mitochondrial dysfunction in aging and that mitochondrial dysfunction is the pacemaker of the decline in neurological performances, which has a determinant role in survival (36).

The present study suggests that the recently discovered mtNOS (19, 21) constitutes an aging marker (34); brain mtNOS has been reported increased during synaptogenesis and spatial memory development (40). The association between mtNOS activity and cellular homeostasis has been called the "pleiotropic effect of mtNOS", and it was suggested that the effect is exerted through NO and H_2O_2 signaling from mitochondria to the cytosol, a process that indicates a high mitochondrial energy charge (10). The marked decrease in mtNOS activity in aged brain mitochondria may constitute a decrease in mitochondrial signaling that does not favor a sustained neuronal homeostasis.

Further studies are required to suggest a threshold for the vitamin E doses that provide beneficial effects in the neurological function in aging mammals, an effect that is likely mediated by the antioxidant properties of α-tocopherol.

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