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High doses of vitamin E improve mitochondrial dysfunction in rat hippocampus and frontal cortex upon aging

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[1]*Departments of Biochemistry and Molecular Biology, and* [2]*Cell Biology and Histology, Faculty of Medicine, University of Cádiz, Cádiz, Spain;* [3]*School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina; and* [4]*Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, California*

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Navarro A, Bandez MJ, Lopez-Cepero JM, Gómez C, Boveris AD, Cadenas E, Boveris A. High doses of vitamin E improve mitochondrial dysfunction in rat hippocampus and frontal cortex upon aging. *Am J Physiol Regul Integr Comp Physiol* 300: R827–R834, 2011. First published November 24, 2010; doi:10.1152/ajpregu.00525.2010.—Rat aging from 4 to 12 mo was accompanied by hippocampus and frontal cortex mitochondrial dysfunction, with decreases of 23 to 53% in tissue and mitochondrial respiration and in the activities of complexes I and IV and of mitochondrial nitric oxide synthase (mtNOS) ($P < 0.02$). In aged rats, the two brain areas showed mitochondria with higher content (35–78%) of oxidation products of phospholipids and proteins and with higher (59–95%) rates of O_2^- and H_2O_2 production ($P < 0.02$). Dietary supplementation with vitamin E (2.0 or 5.0 g/kg of food) from 9 to 12 mo of rat age, restored in a dose-dependent manner, the decreases in tissue and mitochondrial respiration (to 90–96%) and complexes I and IV and mtNOS activities (to 86–88%) of the values of 4-mo-old rats ($P < 0.02$). Vitamin E prevented, by 73–80%, the increases in oxidation products, and by 62–68%, the increases in O_2^- and H_2O_2 production ($P < 0.05$). High resolution histochemistry of cytochrome oxidase in the hippocampal CA1 region showed higher staining in vitamin E-treated rats than in control animals. Aging decreased (19%) hippocampus mitochondrial mass, an effect that was restored by vitamin E. High doses of vitamin E seem to sustain mitochondrial biogenesis in synaptic areas.

brain aging; complex I syndrome; antioxidant protection; hippocampus aging; frontal cortex aging

THE MORE IMPORTANT SYNAPTIC areas (i.e., the gray matter) of mammalian brain in terms of volume are cerebral cortex, hippocampus, cerebellum, and striatum. Other gray matter formations (about 35 in each brain hemisphere) are much smaller in size. In the rat, the four mentioned gray matter main areas provide enough tissue for subcellular fractionation (19, 25) and for the determination of enzyme activities and of biochemical composition (9). The heterogeneity of organization, function, and biochemical mechanisms involved in brain physiology and homeostasis point out to the convenience of studies performed in defined brain areas. The mammalian cerebral cortex is the outermost layer that covers the surface of cerebral hemispheres, and the one corresponding to the frontal area, the frontal cortex, is the site for memory and environmental and self awareness. In humans, frontal cortex includes the specialized areas for language and thought. Hippocampus is the median and bilateral formation of the temporal lobe with memory and cognitive functions and is the brain area that is first damaged in Alzheimer's disease patients associated with memory loss and disorientation. Both, cerebral cortex and hippocampus are known to undergo metabolic and morphological atrophy during aging in rats and in humans (13, 14). Recently, we have reported a mitochondrial dysfunction in rat hippocampus and frontal cortex associated with oxidative damage upon aging (25). The study was performed in rats aged 4, 12, and 20 mo with determinations of mitochondrial respiration, of the enzymatic activities of respiratory complexes and of mitochondrial nitric oxide synthase (mtNOS) in parallel with the determination of the content of oxidation products of proteins and phospholipids, as protein carbonyls and thiobarbituric reactive substance (TBARS) in whole brain, frontal cortex, and hippocampus. In the three brain preparations, respiration and enzymatic activities decreased and oxidation products increased upon aging. Interestingly, the changes were more marked in hippocampus than in frontal cortex, and both were greater than in whole brain. It is then clear that hippocampus and frontal cortex are rat brain areas sensitive to aging in terms of mitochondrial impairment and of oxidative damage, two phenomena that are considered direct causes of a progressively higher rate of apoptosis and of organ atrophy in aging (25).

Our research group also reported a beneficial effect of high doses of vitamin E (5.0 g/kg of food) in aging mice, such as better respiratory functions of whole brain mitochondria in parallel with better performances in neuromuscular coordination and exploratory activities and with an increased survival. Improvements were in the range of 40% for median life span, 24–28% for the performances in the tightrope and T-maze tests, and 40–66% for respiratory and enzymatic activities (24). The rates of electron transfer in complex I and IV correlated positively with successes in the behavioral tests and with life span, suggesting a rate-limiting role for mitochondrial electron transfer and energy generation in brain physiological activity. Such previous observations prompted us to assay the effects of vitamin E (at 2.0 and 5.0 g/kg of food) in rat brain in the sensitive areas of hippocampus and frontal cortex, consid-

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Table 1. *Effect of vitamin E on tissue respiration of rat hippocampus and frontal cortex upon aging*

Condition/Tissue	Oxygen Uptake, ng-at O/min ± g tissue
Control rats, 4 mo	
Hippocampus	1,003 ± 22 (100%)
Frontal cortex	1,004 ± 21 (100%)
Control rats, 12 mo	
Hippocampus	709 ± 18* (71%)
Frontal cortex	772 ± 18* (77%)
Rats, 12 mo, with vitamin E: 2.0 g α-tocopherol acetate/kg food during 3 mo	
Hippocampus	912 ± 20† (91%)
Frontal cortex	906 ± 19† (90%)
Rats, 12 mo, with vitamin E: 5.0 g α-tocopherol acetate/kg food during 3 mo	
Hippocampus	963 ± 21† (96%)
Frontal cortex	944 ± 21† (94%)

Values are means ± SE. *$P < 0.001$, 12-mo-old compared with 4-mo-old rats; †$P < 0.001$, vitamin E compared with control animals at 12 mo of age. ANOVA parameters: $F_{(7,43)} = 28.2$, $P < 0.0001$.

ering as control the effects of aging from 4 to 12 mo of rat age, and as the experimental groups the animals fed the two doses of vitamin E from 9 to 12 mo of age, i.e., during 3 mo, a period that approximately corresponds to three brain mitochondria turnover times ($t_{1/2}$ = 30 days) (29).

The aim of this work was to confirm the beneficial effects of high doses of vitamin E in rodent brain mitochondrial function (22) in the age-sensitive areas of rat hippocampus and frontal cortex (23).

MATERIALS AND METHODS

Animals. Male Wistar rats were fed a standard laboratory animal food (255 ± 11 and 486 ± 15 g, A04 diet; Panlab LS, Barcelona, Spain) containing 30 mg α-tocopherol/kg of food (24), from 4 to 9 mo of age. Control animals were kept on the same diet from 9 to 12 mo of age. Vitamin E-supplemented animals received either 2.0 or 5.0 g *l*-RRR-α-tocopherol acetate/kg of food from 9 to 12 mo of age. At the end point of 12 mo the rat weights were: controls, 572 ± 14 g; vitamin E at 2.0 g/kg, 580 ± 10 g; and vitamin E at 5.0 g/kg, 575 ± 12 g. Control groups (4 and 12 mo) consisted of 30 rats per each group: six rats for whole brain, and 24 rats for brain cortex and hippocampus in pools of four rats. Vitamin E-supplemented groups consisted of 26 rats. Six rats were used to obtain whole brain and 20 rats for the samples of frontal cortex and hippocampus. The animals were grown at the Department of Experimental Animals of the University of Cadiz, housed in groups of three rats per cage and kept at 22 ± 2°C with 12:12-h light-dark cycles and with full access to water and food. Experiments were carried out in accordance with the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society, the Guidelines of the European Union Council (86/609/CEE), and the Spanish regulations (BOE 67/8509-12, 1988) for laboratory animals, and were approved by the Scientific Committee of the University of Cádiz.

Surgical procedure. Rats were killed by decapitation, and the frontal cortex and hippocampus were removed by the Lopez-Cepero and Navarro surgical procedure (25) that yielded 158 ± 8 and 167 ± 9 mg of hippocampi per rat of 4- and 12-mo-old rats. The excised frontal cortex gave 390 ± 15 and 595 ± 18 mg per rat of 4- and 12-mo-old rats. Both brain areas were rapidly washed and immersed in 230 mM mannitol, 70 mM sucrose, 1.0 mM EDTA, and 10 mM Tris·HCl, pH 7.4, at 0°C as previously described (25).

Frontal cortex and hippocampus tissue oxygen consumption. Frontal cortex and hippocampus were sectioned in 1-mm^3 cubes, and the O_2 uptake of 6–8 cubes was determined polarographically with a Clark electrode (Hansatech Instruments) in a 1.5-ml chamber at 30°C, in an air-saturated Krebs medium consisting of: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 5.5 mM glucose, pH 7.3. Respiration was linear with the number of cubes (tissue mass) and during the initial 3–10 min of the measurement and is expressed in ng-at O/min × g tissue (22).

Isolation of mitochondria. Pools of four rats were used for hippocampus and frontal cortex and one rat for whole brain. Mitochondria were isolated from tissues homogenized in 230 mM mannitol, 70 mM sucrose, 1.0 mM EDTA, and 10 mM Tris·HCl, pH 7.40, at a ratio of 9 ml of homogenization medium/g of tissue in a Potter homogenizer with a Teflon pestle by conventional centrifugation as described before (21, 23, 25, 26). Hippocampal and frontal cortex mitochondria

Table 2. *Effects of vitamin E on the respiration of mitochondria isolated from hippocampus, frontal cortex, and whole brain upon rat aging*

Condition/Tissue	Oxygen Uptake, ng-at O/min × mg protein		Respiratory Control
	State 4	State 3	
Substrate: malate-glutamate			
Control rats, 4 mo			
Hippocampus	24 ± 2	103 ± 7 (100%)	4.3 ± 0.2
Frontal cortex	24 ± 2	132 ± 7 (100%)	5.5 ± 0.3
Whole brain	24 ± 2	133 ± 9 (100%)	5.6 ± 0.3
Control rats, 12 mo			
Hippocampus	24 ± 2	65 ± 6* (63%)	2.7 ± 0.2*
Frontal cortex	23 ± 2	94 ± 8* (71%)	4.1 ± 0.2*
Whole brain	23 ± 2	109 ± 9* (81%)	4.7 ± 0.3*
Rats, 12 mo, with vitamin E: 2.0 g α-tocopherol acetate/kg food for 3 mo			
Hippocampus	23 ± 2	94 ± 7† (92%)	4.1 ± 0.2†
Frontal cortex	23 ± 2	119 ± 7† (90%)	5.2 ± 0.2†
Whole brain	23 ± 2	118 ± 8 (88%)	5.1 ± 0.3
Rats, 12 mo, with vitamin E: 5.0 g α-tocopherol acetate/kg food for 3 mo			
Hippocampus	23 ± 2	100 ± 9† (96%)	4.4 ± 0.2†
Frontal cortex	23 ± 2	125 ± 8† (95%)	5.5 ± 0.3†
Whole brain	23 ± 2†	124 ± 9† (92%)	5.4 ± 0.4
Substrate: succinate			
Control rats, 4 mo			
Hippocampus	44 ± 3	180 ± 10 (100%)	4.1 ± 0.2
Frontal cortex	45 ± 3	189 ± 10 (100%)	4.3 ± 0.3
Whole brain	47 ± 4	199 ± 11 (100%)	4.3 ± 0.4
Control rats, 12 mo			
Hippocampus	43 ± 4	135 ± 8* (75%)	3.3 ± 0.2*
Frontal cortex	46 ± 3	151 ± 9* (80%)	3.4 ± 0.2*
Whole brain	46 ± 4	170 ± 11* (85%)	3.8 ± 0.3
Rats, 12 mo, with vitamin E: 2.0 g α-tocopherol acetate/kg food for 3 mo			
Hippocampus	44 ± 4	163 ± 10 (90%)	3.7 ± 0.2
Frontal cortex	45 ± 4	174 ± 10 (92%)	3.9 ± 0.2
Whole brain	46 ± 4	185 ± 11 (93%)	4.0 ± 0.3
Rats, 12 mo, with vitamin E: 5.0 g α-tocopherol acetate/kg food for 3 mo			
Hippocampus	43 ± 4	174 ± 9† (97%)	4.1 ± 0.2†
Frontal cortex	46 ± 4	182 ± 9† (97%)	4.1 ± 0.2†
Whole brain	45 ± 3	192 ± 10 (96%)	4.3 ± 0.2

Values are means ± SE. *$P < 0.02$, 12-mo-old compared with 4-mo-old rats; †$P < 0.02$, rats with vitamin E compared with control animals at 12 mo of age. ANOVA parameters: *1*) for malate-glutamate: state 4, $F_{(11,67)} = 0.11$, not significant (NS); state 3, $F_{(11,67)} = 6.3$, $P < 0.0001$; respiratory control, $F_{(11,67)} = 10.8$, $P < 0.0001$; *2*) for succinate: state 4, $F_{(11,67)} = 0.11$, NS; state 3, $F_{(11,67)} = 3.1$, $P < 0.05$; respiratory control, $F_{(11,67)} = 2.4$, $P < 0.05$.

had 0.24 ± 0.01 and 0.22 ± 0.01 nmol cytochrome aa_3/mg protein in agreement with the classical procedure for isolation of rat brain mitochondria (2). Other procedures were performed as described (25).

Mitochondrial oxygen uptake and mtNOS functional activity. Mitochondrial O_2 uptake was measured with a Clark electrode in a 1.5-ml chamber at 30°C in an air-saturated reaction medium consisting of 230 mM mannitol, 70 mM sucrose, 20 mM Tris·HCl, pH 7.40, 1.0 mM EDTA, 5.0 mM phosphate, 4.0 mM $MgCl_2$, and 0.5–0.7 mg mitochondrial protein/ml, at pH 7.40 (7) as described before (25). Respiratory rates and respiratory control ratios were determined as described before (25). mtNOS functional activity was assayed by the determination of the difference between the rates of state 3 respiration in the presence of *1*) arginine and Cu,Zn-SOD (*a*) and *2*) the NOS inhibitor L-NAME and of hemoglobin (*b*), as described before (25). The difference in oxygen uptake of $a - b$ gives the mtNOS functional activity in inhibiting cytochrome oxidase activity and respiration (24, 38).

Mitochondrial electron transfer activities. The enzyme activities of complexes I, II, and IV were determined spectrophotometrically at 30°C as previously described (23, 25, 26).

Brain mitochondrial content. The content of mitochondria (mitochondrial mass) in rat brain preparations was calculated from the ratio of the activities of cytochrome oxidase in homogenates and in isolated mitochondria and expressed as milligram of mitochondrial protein per gram brain cortex (22, 27, 32).

Spectrophotometric determination of mtNOS activity. Mitochondrial NO production was determined by the oxyhemoglobin (HbO_2) oxidation assay at 30°C (6) in the experimental conditions previously described (25).

Quantification of O_2^- production. The superoxide anion-dependent oxidation of epinephrine to adrenochrome (20) was determined in mitochondrial fragments by following the spectrophotometric changes at 485–575 nm ($\varepsilon = 2.96\ mM^{-1}\cdot cm^{-1}$) at 30°C (spectrophotometer 8453; Agilent, Palo Alto, CA). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 30 mM Tris·HCl (pH 7.4), 0.5–0.8 mg protein/ml, 20 μM NADH, 1 μM rotenone; 0.1 μM catalase, and 1 mM epinephrine. As a control of assay specificity, 2 μM Cu,Zn-SOD inhibited the reaction over 92%.

Determination of hydrogen peroxide generation. Production of H_2O_2 was determined in mitochondria (0.3–0.5 mg protein/ml) by the scopoletin-horseradish peroxidase (HRP) method, following the decrease in fluorescence intensity at 365–450 nm (λexc-λem) at 30°C (5). The reaction mixture contained 0.23 M mannitol, 0.07 M sucrose, 30 mM Tris·HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 20 μM NADH, 1 μM rotenone, and 0.3 μM Cu,Zn-SOD.

Biochemical markers of oxidative damage. Protein carbonyls and TBARS were determined in mitochondrial membranes by the assays of Fraga et al. (15) and Oliver et al. (28), modified as described (21) and expressed in nanomoles per milligram of mitochondrial protein. Although both assays are not specific, they have been useful in our experience, and their increases, taken as oxidative damage, correlate with the loss of mitochondrial enzymatic activities (21).

Histochemical determination of complex IV. One control and one vitamin E-supplemented hippocampal samples were simultaneously processed. Complex IV was assayed by the classical diaminobenzidine Seligman's method (37) with heavy metal intensification followed by enhancement with ammoniacal silver (25, 36). A silver carbonate solution was used for 1 min at room temperature on floating sections, then washed and reduced in neutral 10% formaldehyde followed by gold toning (0.166 mg gold chloride/100 ml) for 10 min followed by hyposulfite fixation and washing. High-resolution digital microphotographs were obtained at ×100 with an immersion objective in a Nikon Optiphot-2 microscope with a DXM1200F digital camera. Each microscopic field was imaged as a z-stack series and used to reconstruct a stereopair image of mitochondrial distribution. Single z-planes were surface-plotted as 3D images to visualize mitochondrial size and density distribution. Image processing and analysis were performed with Image-J software (National Institutes of Health, Bethesda, MD) (1).

Statistics. The differences between groups were analyzed by the Student-Newman-Keuls as post hoc test after significant one-way ANOVA. A P value of < 0.05 was considered statistically significant. Statistical analyses were carried out using the statistical package SPSS 11.5 for Windows.

RESULTS

Rat aging from 4 mo (young adult) to 12 mo of age (old adult) was associated with a clear (23–29%) decrease in tissue respiration in hippocampus and frontal cortex (Table 1). Di-

Table 3. *Effects of vitamin E on the enzymatic activity of mitochondrial complexes I, II, and IV and the enzymatic activity of mitochondrial nitric oxide synthase (mtNOS) in mitochondria isolated from hippocampus, frontal cortex, and whole brain upon rat aging*

Condition/Tissue	Complex I	Complex II	Complex IV	mtNOS
Control rats, 4 mo				
Hippocampus	300 ± 13 (100%)	119 ± 9	180 ± 9 (100%)	2.20 ± 0.05 (100%)
Frontal cortex	320 ± 12 (100%)	125 ± 9	133 ± 9 (100%)	1.50 ± 0.06 (100%)
Whole brain	324 ± 14 (100%)	122 ± 9	152 ± 9 (100%)	1.50 ± 0.06 (100%)
Control rats, 12 mo				
Hippocampus	162 ± 8* (54%)	115 ± 9	85 ± 8* (47%)	1.42 ± 0.06* (69%)
Frontal cortex	246 ± 9* (77%)	119 ± 9	96 ± 8* (72%)	1.14 ± 0.04* (76%)
Whole brain	259 ± 11* (80%)	129 ± 9	105 ± 9* (69%)	1.08 ± 0.04* (72%)
Rats, 12 mo, with vitamin E: 2.0 g α-tocopherol acetate/kg food during 3 mo				
Hippocampus	270 ± 9† (90%)	117 ± 9	127 ± 9† (71%)	1.90 ± 0.04† (86%)
Frontal cortex	272 ± 10 (85%)	118 ± 9	100 ± 9 (75%)	1.23 ± 0.04 (82%)
Whole brain	259 ± 12 (80%)	124 ± 9	110 ± 9 (72%)	1.25 ± 0.04† (83%)
Rats, 12 mo, with vitamin E: 5.0 g α-tocopherol acetate/kg food during 3 mo				
Hippocampus	285 ± 12† (95%)	121 ± 9	190 ± 9† (105%)	2.05 ± 0.07† (93%)
Frontal cortex	294 ± 13† (92%)	118 ± 9	120 ± 9† (90%)	1.33 ± 0.04† (89%)
Whole brain	288 ± 12 (89%)	125 ± 9	134 ± 9† (88%)	1.31 ± 0.05† (87%)

Values are means ± SE. Complex I, complex II, and complex IV activities are expressed in nmol cytochrome *c* (reduced or oxidized)/min × mg protein; mtNOS expressed in nmol NO/min × mg protein. *$P < 0.02$, 12-mo-old compared with 4-mo-old rats; †$P < 0.02$, rats with vitamin E compared with control rats at 12 mo of age. ANOVA parameters: complex I, $F_{(11,67)} = 14.3$, $P < 0.0001$; complex II, $F_{(11,67)} = 0.3$, NS; complex IV, $F_{(11,67)} = 13.8$, $P < 0.0001$; and mtNOS, $F_{(11,67)} = 47.5$, $P < 0.0001$.

etary supplementation with high doses of vitamin E during 3 mo (from 9 to 12 mo of age) was effective in preventing the decrease in hippocampus and frontal cortex respiration. Vitamin E, at 2.0 and 5.0 g/kg of food, restored hippocampus respiration to 94 and 96% and frontal cortex respiration to 90 and 91% of the corresponding tissue respiration in young (4-mo-old) rats.

Similarly, rat aging from 4 to 12 mo showed a clear (29–27%) decrease in the state 3 rate of mitochondrial O_2 uptake, the condition of active respiration and ATP synthesis, in hippocampal and frontal cortex mitochondria with malate-glutamate as substrate (Table 2) in agreement with our previous report (25). The dietary supplementation with vitamin E was effective in preventing the decrease in mitochondrial respiration in state 3: at 2.0 and 5.0 g/kg of food, hippocampal mitochondrial respiration was at 92 and 96% of the values for young rats, and frontal cortex mitochondrial respiration was at 90 and 95% of the rates for 4-mo-old rats (Table 2). Hippocampal and frontal cortex mitochondria were more sensitive than whole brain mitochondria to the inhibitory effect of aging and to the restoration due to vitamin E effect (Table 2). Although similar, the phenomena were less marked with succinate as substrate (Table 2), implying that complex I (NADH-ubiquinone reductase) is the selectively most sensitive target for both aging-associated damage and vitamin E restoration.

The selective impairment of complex I and the protective effect of vitamin E were confirmed by direct determination of complex I activity; the 53 and 35% decreased activity of complex I associated with aging in hippocampal and frontal cortex mitochondria was effectively restored by vitamin E supplementation with conservation of the enzymatic activities to 95 and 96% of the level of young animals (Table 3).

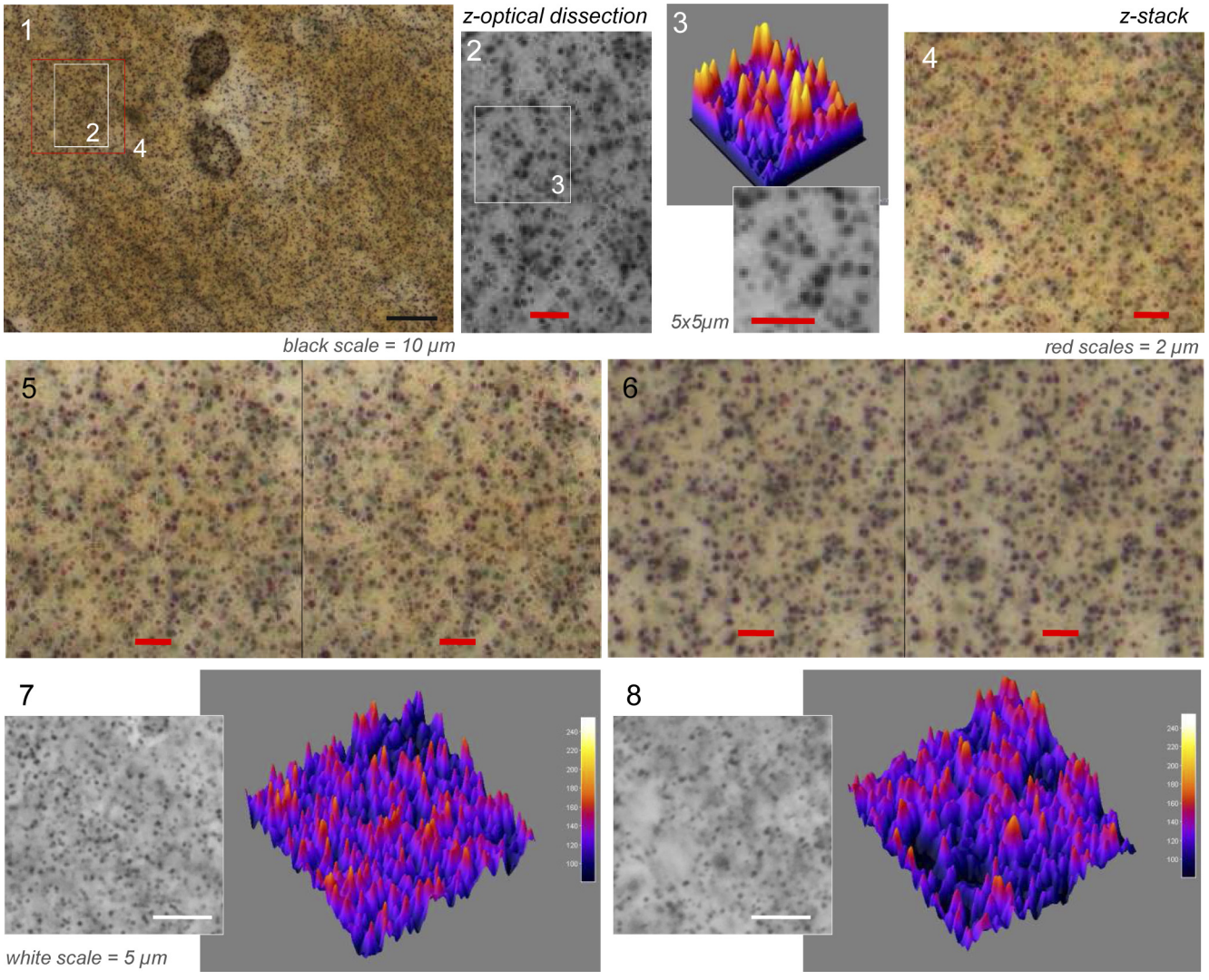

Fig. 1. Mitochondrial distribution in the stratum radiatum neuropil of the CA1 hippocampus region. Cytochrome oxidase histochemistry with silver enhancement of diaminobenzidine deposits. Stratum radiatum of CA1 hippocampal field (*1*) of a 12-mo-old control rat. Z-projection of a z-stack shows mitochondria and 2 endothelial oval cell bodies (*center*). Sample areas are selected. A single-focused z-plane (*2*) makes an optical dissection showing focused and over/under focused organelles in an 8-bit image. Organelle resolution is assessed by surface plotting of gray levels (*3*) from the square selected area in (*2*). The same sampling area is shown (*4*) and include a few thousand mitochondria. Two stereo pairs from 12-mo-old control animals (*5*) and the same age vitamin E supplemented rats (*6*) show subtle differences in mitochondrial size, reactivity, and spatial pattern. Single-focused z-planes from each of the above fields are imaged as surface plots in *7* and *8*. Control rats show a relatively low-stained, small-sized mitochondria (*7*), and vitamin E-supplemented rats show increased staining in size and density (*8*).

Table 4. *Effect of vitamin E on the mtNOS functional activity of hippocampal and frontal cortex mitochondria upon rat aging*

Condition/Tissue	Oxygen Uptake, ng-at O/min × mg protein			mtNOS Functional Activity, ng-at O/min × mg protein (*c–b*)
	State 3 (*a*)	State 3 + arginine (*b*)	State 3 + L-NAME (*c*)	
Control rats, 4 mo				
Hippocampus	103 ± 7	85 ± 7	131 ± 10	46 ± 4 (100%)
Frontal cortex	132 ± 7	120 ± 9	164 ± 11	44 ± 5 (100%)
Whole brain	135 ± 8	124 ± 9	178 ± 10	54 ± 4 (100%)
Control rats, 12 mo				
Hippocampus	65 ± 6	62 ± 6	84 ± 8	22 ± 3* (48%)
Frontal cortex	94 ± 8	84 ± 7	111 ± 9	27 ± 2* (61%)
Whole brain	109 ± 7	98 ± 8	140 ± 10	42 ± 5* (78%)
Rats, 12 mo, with vitamin E: 2.0 g α-tocopherol acetate/kg food during 3 mo				
Hippocampus	94 ± 7	78 ± 8	108 ± 8	30 ± 3 (65%)
Frontal cortex	119 ± 7	93 ± 8	131 ± 9	38 ± 4† (86%)
Whole brain	118 ± 8	92 ± 8	132 ± 9	40 ± 4 (74%)
Rats, 12 mo, with vitamin E: 5.0 g α-tocopherol acetate/kg food during 3 mo				
Hippocampus	100 ± 8	84 ± 8	129 ± 9	45 ± 4† (98%)
Frontal cortex	125 ± 9	114 ± 9	154 ± 10	40 ± 4† (91%)
Whole brain	124 ± 9	113 ± 9	160 ± 10	47 ± 4 (87%)

Values are means ± SE. L-NAME, N^G-nitro-L-arginine methyl ester. *$P < 0.02$, mtNOS functional activity of 12-mo-old compared with 4-mo-old rats; †$P < 0.02$, mtNOS functional activity from rats with vitamin E compared with control rats at 12 mo of age. ANOVA parameter: mtNOS $F_{(11,67)} = 6.5$, $P < 0.0001$.

Similarly, complex IV (cytochrome oxidase) and mtNOS activities of hippocampal and frontal cortex mitochondria were decreased in the aging process by 23 and 46% and the enzymatic activities were largely (85–95%) restored by vitamin E supplementation (Table 3).

These two effects, impairing effect of aging and beneficial effects of vitamin E in the hippocampus were also supported by the histochemical determination of cytochrome oxidase activity. The Seligman's histochemical method with silver enhancement was able to show, at single-organelle resolution, that the neuropil in striatum radiatum exhibits mainly synaptic mitochondria that are homogeneous and with reduced size in control 12-mo-old rats, whereas that the same organelles show a greater cytochrome oxidase histochemical reactivity with higher staining and size variation in vitamin E-supplemented animals (Fig. 1, see Fig. 1 legend and *plates 7* and *8*), in agreement with the direct determination of biochemical cytochrome oxidase activity described in Table 3.

Again, the two above-mentioned effects, impairing effect of aging and beneficial effects of vitamin E, were observed in mtNOS functional activity in hippocampal and frontal cortex mitochondria with malate-glutamate as substrate (Table 4).

Vitamin E supplementation for 3 mo (from 9 to 12 mo of age) was also able to restore the content of oxidation products (TBARS and protein carbonyls) observed in mitochondria from hippocampus and frontal cortex of rats 4 mo of age (Table 5).

The production of superoxide radical and hydrogen peroxide was assayed in mitochondrial fragments in the presence of NADH and rotenone, a condition that allows the determination of the specific production of both species by complex I (10). Rat aging produced a marked increase, in the range of 30–95%, in the generation of O_2^- and H_2O_2, by complex I in the three mitochondrial preparations (hippocampus, frontal cortex, and whole brain). Vitamin E was effective in restoring the activities to values close to the ones of young rats.

A determination of the mitochondrial mass in the two brain areas and in the whole brain upon aging showed that only the hippocampus showed a significant decrease (19%) in mitochondrial mass upon aging (from 11.1 to 9.0 mg protein/g hippocampus) (Table 7). Vitamin E supplementation for 3 mo at 2.0 and 5.0 g/kg was able to restore hippocampal mitochondrial mass to 9.6 and 11.0 mg/g hippocampus (Table 7).

Table 5. *Effects of vitamin E on the content of phospholipid and protein oxidation products in mitochondria isolated from hippocampus and frontal cortex upon rat aging*

Condition/Tissue	Phospholipid Oxidation	Protein Carbonyls
Control rats, 4 mo		
Hippocampus	1.4 ± 0.2 (100%)	18 ± 1 (100%)
Frontal cortex	1.4 ± 0.2 (100%)	29 ± 3 (100%)
Whole brain	1.3 ± 0.2 (100%)	20 ± 2 (100%)
Control rats, 12 mo		
Hippocampus	2.1 ± 0.2* (150%)	32 ± 3* (178%)
Frontal cortex	2.0 ± 0.2* (143%)	39 ± 3* (134%)
Whole brain	1.8 ± 0.2* (138%)	30 ± 2* (150%)
Rats, 12 mo, with vitamin E: 2.0 g α-tocopherol acetate/kg food during 3 mo		
Hippocampus	1.5 ± 0.2 (107%)	25 ± 2 (139%)
Frontal cortex	1.7 ± 0.2 (121%)	35 ± 3† (121%)
Whole brain	1.4 ± 0.2 (108%)	26 ± 2 (130%)
Rats, 12 mo, with vitamin E: 5.0 g α-tocopherol acetate/kg food during 3 mo		
Hippocampus	1.4 ± 0.2† (100%)	19 ± 1† (106%)
Frontal cortex	1.5 ± 0.2† (107%)	30 ± 3† (103%)
Whole brain	1.4 ± 0.2 (108%)	23 ± 2 (115%)

Values are means ± SE. *$P < 0.02$, 12-mo-old as compared with 4-mo-old rats; †$P < 0.02$, rats with vitamin E compared with control rats at 12 mo of age. Phospholipid oxidation as thiobarbituric reactive substance (TBARS). ANOVA parameters: TBARS, $F_{(11,67)} = 1.9$, $P < 0.05$; protein carbonyls, $F_{(11,67)} = 46.7$, $P < 0.0001$.

Table 6. *Effect of vitamin E on the production of superoxide radical and hydrogen peroxide by complex I (NADH- and rotenone-supplemented) in hippocampal, frontal cortex, and whole brain mitochondrial fragments upon rat aging*

Age/Tissue	Superoxide Radical, nmol O_2^-/min × mg protein	Hydrogen Peroxide, nmol H_2O_2/min × mg protein
Control rats, 4 mo		
Hippocampus	1.7 ± 0.2 (100%)	0.71 ± 0.06 (100%)
Frontal cortex	1.8 ± 0.2 (100%)	0.78 ± 0.06 (100%)
Whole brain	2.1 ± 0.2 (100%)	0.82 ± 0.06 (100%)
Control rats, 12 mo		
Hippocampus	3.3 ± 0.2* (194%)	1.41 ± 0.09* (199%)
Frontal cortex	3.5 ± 0.2* (195%)	1.24 ± 0.08* (159%)
Whole brain	2.7 ± 0.2* (129%)	1.07 ± 0.07* (130%)
Rats, 12 mo, with vitamin E: 2.0 g α-tocopherol acetate/kg food during 3 mo		
Hippocampus	2.8 ± 0.2† (165%)	0.96 ± 0.07† (135%)
Frontal cortex	2.9 ± 0.2† (161%)	0.97 ± 0.08† (124%)
Whole brain	2.5 ± 0.2 (119%)	0.93 ± 0.08 (113%)
Rats, 12 mo, with vitamin E: 5.0 g α-tocopherol acetate/kg food during 3 mo		
Hippocampus	1.9 ± 0.2† (112%)	0.80 ± 0.06† (113%)
Frontal cortex	2.5 ± 0.2† (139%)	0.87 ± 0.08† (112%)
Whole brain	2.3 ± 0.2† (110%)	0.87 ± 0.07† (106%)

Values are means ± SE. *$P < 0.02$, 12-mo-old compared with 4-mo-old rats; †$P < 0.05$, rats with vitamin E compared with control rats at 12 mo of age. ANOVA parameters: superoxide radical, $F_{(11,67)} = 9.2$, $P < 0.0001$; hydrogen peroxide, $F_{(11,67)} = 7.9$, $P < 0.0001$.

DISCUSSION

Isolation of frontal cortex and hippocampal mitochondria is a recent achievement (25) considering the 485 ± 20 mg of frontal cortex and the 175 ± 20 mg of left and right hippocampi obtained from a single rat. The hippocampal and frontal cortex preparations are comparable in terms of mitochondrial quality, i.e., the rate of state 3 respiration and the respiratory control ratio, to the classic and reference values for rat whole brain mitochondria (12). Upon aging, frontal cortex and hippocampal mitochondria show a decreased rate of respiration, especially marked with NAD-dependent substrates, and decreased enzymatic activities of complexes I and IV associated with an increase in the content of oxidation products (TBARS and protein carbonyls) (25). Of course, cell fractionation of frontal cerebral cortex and hippocampus does not discriminate between neurons and glia and fails to provide an identification of the described mitochondrial impairment with the fine and selective age-associated damage in the pyramidal neurons. However, the preparations of hippocampal and frontal cortex mitochondria are constituted by a majority of neuronal soma mitochondria with a minor fraction of axonal mitochondria and are highly efficient in identifying a tissue mitochondrial failure associated with a higher content of phospholipid and protein oxidation products.

The impairment of mitochondrial respiration observed in hippocampus, frontal cortex, and whole brain could be explained by an oxidative modification of electron transfer at complex I that leads to a loss of mitochondrial respiratory function. It is currently accepted that mitochondrial complex I is particularly sensitive to inactivation by oxygen free radicals and reactive nitrogen species. This special characteristic is frequently referred as complex I syndrome, with the symptoms of reduced mitochondrial respiration with malate-glutamate and ADP and of reduced complex I activity. This complex I syndrome has been observed in aging (8), in ischemia-reperfusion (16), in Parkinson's disease, and in other neurodegenerative diseases (11, 22, 33–35), and in this study, with the addition of the increased rates of production of O_2^- and H_2O_2 by complex I.

The molecular mechanism involved in the inactivation of complex I is likely accounted for by the sum of $ONOO^-$

Table 7. *Effect of vitamin E on the mitochondrial content (mitochondrial mass) of hippocampus, frontal cortex, and whole brain upon rat aging*

Condition/Tissue	Cytochrome Oxidase Activity		Mitochondrial Mass, mg protein/g organ
	Homogenate, nmol cyt c^{2+}/min × g organ	Mitochondria, nmol cyt c^{2+}/min × mg protein	
Control rats, 4 mo			
Hippocampus	1,998 ± 118	180 ± 9	11.1 ± 0.4 (100%)
Frontal cortex	1,835 ± 110	133 ± 9	13.8 ± 0.4 (100%)
Whole brain	1,702 ± 110	152 ± 9	11.2 ± 0.4 (100%)
Control rats, 12 mo			
Hippocampus	765 ± 72	85 ± 8	9.0 ± 0.4* (81%)
Frontal cortex	1,300 ± 100	96 ± 8	13.5 ± 0.4 (98%)
Whole brain	1,165 ± 91	105 ± 9	11.1 ± 0.4 (99%)
Rats, 12 mo, with vitamin E: 2.0 g α-tocopherol acetate/kg food during 3 mo			
Hippocampus	1,219 ± 90	127 ± 9	9.7 ± 0.4 (87%)
Frontal cortex	1,360 ± 90	100 ± 9	13.6 ± 0.4 (99%)
Whole brain	1,220 ± 90	110 ± 9	11.0 ± 0.4 (98%)
Rats, 12 mo, with vitamin E: 5.0 g α-tocopherol acetate/kg food during 3 mo			
Hippocampus	2,090 ± 130†	190 ± 9†	11.0 ± 0.4† (99%)
Frontal cortex	1,644 ± 110	120 ± 9	13.7 ± 0.4 (99%)
Whole brain	1,502 ± 110	134 ± 9	11.2 ± 0.4 (100%)

Values are means ± SE. Tissue mitochondrial mass was calculated from the ratios of cytochrome oxidase activities in the homogenate and in isolated mitochondria. *$P < 0.05$, 12-mo-old compared with 4-mo-old rats; †$P < 0.05$, rats with vitamin E compared with control animals at 12 mo of age. ANOVA parameter for mitochondrial mass $F_{(11,67)} = 16.0$, $P < 0.001$.

mediated reactions, reactions with the free radicals intermediates of the lipid peroxidation process (mainly ROO·), and amine-aldehyde adduction reactions. It is now understood that the three process above mentioned alter the native noncovalent polypeptide interactions of complex I and promote synergistically protein damage and inactivation by shifting the noncovalent bonding to covalent cross linking (18). Complex I oxidative protein damage has also been considered the result of protein modification by reaction with malonaldehyde and 4-HO-nonenal (18, 30). It may be hypothesized that protein damage in the subunits of complexes I and IV follows to free radical-mediated cross-linking and inactivation. The subunits that are normally held together by noncovalent forces are shifted to covalent cross-linking after reaction with the hydroperoxyl radicals (ROO·) and the stable aldehydes produced during the lipid peroxidation process.

The hypothesis that cumulative free radical-mediated protein damage is the chemical basis of respiratory complexes I and IV inactivation (3) offers the experimental approach of the chronic use of vitamin E, as an antioxidant for the lipid phase of the inner mitochondrial membrane, for the prevention of the mitochondrial damage associated with aging. The adduction reactions of malonaldehyde and 4-HO-nonenal with protein evolve to stable advanced lipid peroxidation products (31) and protein carbonyls (24, 25). There is now substantial evidence that the processes that alter the native polypeptide noncovalent interaction promote synergistically protein damage and inactivation (30). The observed protective effect of vitamin E in preserving tissue and mitochondrial active respirations certainly supports the hypothesis of a free radical-mediated process with protein oxidation and inactivation in aging. The current status of knowledge is that mitochondria accumulate oxidized products of lipids, proteins, and DNA oxidation as a linear function of time and of respiration. It is postulated that the increased content of oxidation products leads to a decrease in the rate of mitochondrial turnover and biogenesis and to a homeostatic more oxidized state that is closer and prone to the triggering level of apoptosis.

The used doses of vitamin E in rats (2.0 and 5.0 g/kg of rat food) and the daily intake of food by 12-mo-old rats (28 g/day) correspond in terms of milligrams of vitamin E per kilojoules of basal metabolic rate ($BMR = A \times weight^{2/3}$) (39) to human daily doses of 1,390 and 3,475 mg of vitamin E.

The beneficial effect of high doses of vitamin E on rat hippocampus and frontal cortex mitochondria (present study) and on mice neurological performance and survival (22) is to be taken into account in the controversy on the use of vitamin E supplementation in humans. The claim that vitamin E supplementation increases human mortality, based on meta analysis (4), is challenged by the clinical evidence that vitamin E supplements are safe at high intakes (17) and by the reported effects of vitamins E and C in the reduction of prevalence and incidence of Alzheimer disease in an elderly population (40).

High-resolution histochemistry at the level of single mitochondria reveals that 12-mo-old rats have hippocampal dendritic fields with relatively reduced staining intensity for cytochrome oxidase in hippocampus synaptic-rich areas, as described before for mouse brain (25) and in agreement with the reduced cytochrome oxidase activity in hippocampus and frontal cortex (Table 3 and Ref. 25). The animals of the same age, but treated for 3 mo with vitamin E showed increased histochemical staining and complex IV activity (Table 3). The interpretation is that vitamin E leads to sustained mitochondrial biogenesis with increased hippocampal mitochondrial mass. The low mitochondrial oxidative damage under vitamin E supplementation supports the concept of a faster mitochondrial turnover and biogenesis in hippocampus and frontal cortex.

Perspectives and Significance

High doses of vitamin E are effective in preventing the decline in brain mitochondrial function in rodents. The beneficial effect in mouse correlated with a better neurological function and with increased survival. In rats, the effect was more marked in hippocampal and frontal cortex mitochondria than in whole brain mitochondria. Vitamin E, its derivatives, and other antioxidants seem to be potential therapeutic agents for the treatment and prevention of brain dysfunction in aging and neurodegenerative diseases with the aim in the enhancement of mitochondrial biogenesis and turnover (Table 6).

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DISCLOSURES

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