Regional responses in antioxidant system to exercise training and dietary Vitamin E in aging rat brain

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Received 3 February 2003; received in revised form 24 April 2003; accepted 8 May 2003

Abstract

We have evaluated the effect of exercise, Vitamin E and a combination of both on the antioxidant enzymes (AOEs)—superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) along with the products of lipid peroxidation (LP)—malondialdehyde (MDA) and lipofuscin-like auto fluorescent substances (LF-like AFS) in discrete brain regions of rats of 4 (young adults), 8 (old adults), 12 (middle-age) and 22 months (mos) old of age. Hippocampus (HC) showed greater increase in GSH-Px activity than cerebral cortex (CC) to exercise and Vitamin E and was irrespective of the age. A combination of both was effective in the CC of all age groups but not in the supplemented sedentary of 12- and 22-mo-olds. CAT activity increased significantly in the HC of supplemented and trained rats but not in the combination group of any age. SOD increased in both the regions of supplemented trainees. However, old were more benefited in terms of maximal elevation in the HC. Vitamin E reduced MDA content in both regions of adult. LF-like AFS decreased significantly in supplemented sedentary and trainees of all ages. Our results demonstrate that an age-related deficit in AOEs in the CC and HC can be overcome through Vitamin E plus exercise, and further suggests the rationale for looking at these markers of oxidative stress in several age-related neuronal diseases.

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Keywords: Aging; Cerebral cortex; Exercise; Hippocampus; Rat; Vitamin E

1. Introduction

Under normal conditions, the brain is susceptible to oxidative damage due to its high oxygen consumption rate and high levels of polyunsaturated fatty acids (PUFA) and, paradoxically low levels of defense mechanisms exists against the toxicity of free radicals. The antioxidant system (AOS) spans over a wide range of mechanisms such as enzymic AOS which includes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) to small molecular weight compounds such as Vitamin E and glutathione though in relatively low concentrations [6,32] when compared to other organs. Various investigators have determined the effects of aging [17] and exercise [33] on the antioxidant enzymes (AOEs) in the brain of rodents. Although physical exercise is beneficial to the body, it can generate free radicals [24,30] in the untrained. Most of the aforementioned studies have reported either the effects of exercise on the antioxidant bioenzymes in any one age group or only on the effect of the antioxidant adjuncts such as Vitamin E, carotene or Vitamin C in the brain of rodents and none have attempted to look in to the interaction of exercise and dietary antioxidant on the enzymatic antioxidants in the different regions of the aging brain. This is very critical since free radical generation, and detoxification mechanisms in specific regions of the brain will probably fluctuate over the different phases of life span as summarized from in vivo [9] and in vitro [15] studies. These radicals can be crucial causative factors in the impairment of learning and memory processes associated with aging. The present study was based on the following hypotheses: (1) although swim training of moderate intensity and duration, when initiated at various ages, may ameliorate the rats’ defense system in the brain, it by itself may not prove beneficial with advancing age; (2) dietary Vitamin E, when supplemented to animals may be a more effective means of curtailing free radical damage; (3) the extent of protection offered by dietary Vitamin E and exercise training may far outweigh the individual preventive measures with advancing age. The above hypotheses were analyzed through a comprehensive determination of various parameters related to oxidative stress (OS), e.g. SOD, GSH-Px and CAT activities, and markers of cellular peroxidation such as malondialdehyde (MDA) and
lipofuscin-like (LF) auto fluorescent substances (AFS) in the CC and HC that are sites for learning and memory. We have examined rats of four different ages representing young adults (4 months), old adults (8 months), middle-aged (12 months) and old (22 months) encompassing the entire life span.

2. Materials and methods

The present study was approved by the Institutional Animal Ethics Committee (IAEC), Bangalore University, Bangalore, India.

2.1. Animal care and training protocol

Male albino rats of Wistar strain of 4 months (mos) old were obtained from the central animal facility, IISc, Bangalore and were maintained until they were 8, 12, and 22 mos in a clean rodent room. The maximum length of life of male Wistar rats in our animal colonies has been 30 months with the median length of 15.5 months. Experiments were completed taking into account the entire age-scale encompassing the young adult, old adult, middle-aged and the old. Animals were housed 2–3 per cage in cages that were fitted with stainless-steel wire-mesh bottoms and maintained at a temperature of 28 °C, relative humidity of 77.5±1% and under a daily photoperiod of 12 h light and 12 h dark cycle. The animals were fed with lab chow (Lipton India Ltd., Bangalore) and tap water ad libitum. Rats were assigned to (A) two sedentary groups—controls without (SE-C, N) and with Vitamin E supplementation (SE-C, +E), and, (B) two swim-trained groups—swim trainees on a normal diet (SW-T,N) and swim trainees on Vitamin E supplementation (SW-T, +E). Training protocol was similar to the protocol as described earlier [4]. Briefly, rats were made to swim with 3% of their body weight tied to their tails. Initially they were made to exercise for 5 min per day with a progressive increase to 30 min per day for a total training period of 12 weeks with five training days per week. Two groups of animals received a daily oral supplementation of 50lU/kg of α-tocopherol, throughout the training period. The group SE-C (N) under (A) was restricted to cage activity.

2.1.1. Tissue preparation

Rats in all groups were anaesthetized with ether and sacrificed between 10:00 and 11:00 a.m. on the same day. The brains were collected on ice and cerebral cortex (CC) and hippocampus (HC) were isolated [11], weighed and rinsed several times in ice-cold Tyrodes medium. The regions were homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The resulting homogenates were centrifuged at 1000 rpm for 15 min at 4 °C (SUPERSPIN, Plastocrafts, India). The supernatant was used for analytical procedures.

2.1.2. Analytical procedures

2.1.2.1. Chemicals and solvents. α-Tocopherol, epinephrine, thiobarbituric acid, pyridine, Triton X-100, r-butyl hydrogen peroxide, GSH, GR, NADPH and BSA were obtained from Sigma Chemicals (St. Louis, MO). All organic solvents were of spectral grade and general chemicals were of reagent grade.

2.1.2.2. Enzyme assays. Glutathione peroxidase (GSH-Px, EC 1.11.1.9). GSH-Px activity was measured at 37 °C by the method of Flohe and Gunzler [10]. The reaction mixture consisted of 500 μl of phosphate buffer, 100 μl of 0.01 M reduced glutathione (GSH), 100 μl of 1.5 mM NADPH and 100 μl of GR (0.24 U). One hundred microliters of tissue extract was added to the reaction mixture and incubated at 37 °C for 10 min. Fifty microliters of 12 mM r-butyl hydroperoxide was added to 450 μl of tissue reaction mixture and measured at 340 nm for 180 s in a spectrophotometer (ELICO, Model SL 159). A molar absorptivity of 6.22 × 10³ M cm⁻¹ was used to determine enzyme activity. One unit of activity is equal to nM NADPH oxidized per minute per mg protein.

2.1.2.3. Superoxide dismutase (SOD, EC 1.15.1.1.). SOD activity was determined at room temperature, according to the method of Misra and Fridovich [20]. Tissue extract (100 μl) was added to 880 μl carbonate buffer of 0.05 M, pH 10.2, 0.1 M EDTA. Twenty microliters of 30 mM epinephrine in 0.05% acetic acid was added to the mixture and absorbance was followed for 4 min at 480 nm in a spectrophotometer. The amount of enzyme that results in 50% inhibition of epinephrine autooxidation is defined as one unit.

2.1.2.4. Catalase (CAT, EC 1.11.1.6.). CAT activity was determined at room temperature, according to the method of Aebi [1]. Briefly, 100 μl of tissue extract with an equal volume of absolute alcohol was incubated for 30 min in ice-bath for degradation of inactive CAT-H₂O₂ complex II to release active CAT enzyme. After 30 min on ice the tubes were brought back to room temperature and then 10 μl of Triton X-100 was added. In a cuvette containing 200 μl of phosphate buffer and 50 μl of tissue extract, 250 μl of 0.066 M H₂O₂ in phosphate buffer was added and decrease in absorbance was read at 240 nm for 30 s in a spectrophotometer. A molar absorptivity of 43.6 M cm⁻¹ was used to determine CAT activity, one unit of which is equal to the moles of H₂O₂ degraded per minute per mg protein.

2.1.2.5. Lipid peroxidation (LP). This assay was used to analyze malondialdehyde (MDA) by the method of Ohkawa et. al. [25] using 1,1,3,3-tetraethoxypropane (TTP) as the standard. LP was expressed as n moles MDA per mg protein.

2.1.2.6. Measurement of lipofuscin (LF)-like autofluorescent substances (AFS). Fluorescent substances in the brain regions were measured by the method of Shedahl and Tappel.
[29]. Briefly, tissue homogenates were prepared in 2:1 (v/v) chloroform:methanol and centrifuged at 3000 rpm for 10 min at 4 °C. The pellet was suspended in the same solvent mixture and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were combined and re-centrifuged at 6000 rpm for 10 min. The lower chloroform layer was aspirated and fluorescence was measured at an excitation wavelength of 366 nm and emission wavelength of 485 nm using quinine sulfate in 0.1N sulfuric acid in a spectrofluorimeter (Genway, Model 6299, UK). Auto fluorescence intensity (AFI) was expressed as units per 100 mg tissue.

2.1.2.7. Measurement of protein. Protein concentration was estimated by the method of Lowry et al. [18].

2.1.3. Statistical analyses

All the data were expressed as means ± S.E. and were analyzed within a three-factor analysis of variance (ANOVA) between age, groups, and regions. When a significant F-ratio was found, Duncan’s multiple range tests (DMRT) were used to assess the differences between group means. Probability values (P) < 0.05 were considered significant [32].

3. Results

3.1. Tissue antioxidants

GSH-Px activity in the hippocampus of supplemented sedentary rats increased significantly in young and old adult where as the middle-aged and old showed significant increase in the supplemented trainees (SW-T, E 96% in middle-aged and 61% in the old (P < 0.05) of their respective SE-C (N). Four-month-olds showed significant increases in the CC and HC in response to exercise training as well as Vitamin E. However, maximum increase was seen when exercise and Vitamin E were combined. The extent of increase in GSH-Px activity was greater in the HC (91%) than in the CC (66%) of old supplemented trainees. The same trend was seen between the regions i.e. HC exhibiting greater increase than CC in the adult and middle-aged too (Table 1).

In 4-mo-olds, a significant increase in SOD activity was seen in the HC of supplemented trainees (31% of SE-C, N) (P < 0.05) and this trend was no different from the unsupplemented trainees (32% of SE-C, N) (P < 0.05). CC showed 33 and 22% increase of (SE-C, N, P < 0.05) in the supplemented and unsupplemented trainees respectively. In the 8-mo-olds, significant increases (P < 0.05) were seen in the CC of all the groups and this was with respect to the unsupplemented sedentary. Among the middle-aged, exercise training and Vitamin E significantly increased the SOD in both the regions (HC, 35.6% in SW-T, E; CC, 33.8%, SW-T, E of SE-C, N (P < 0.05). Sedentary when supplemented with Vitamin E showed significant increase in enzyme activity in the HC (23.0% of SE-C, N (P < 0.05) and CC (33.0% of SE-C, N). Old supplemented trainees showed significant increase than CC in the adult and middle-aged too (Table 1).

In 4-mo-olds, a significant increase in SOD activity was seen between the regions i.e. HC exhibiting greater increase than CC in the adult whereas the middle-aged and old showed significant increase when exercise and Vitamin E were combined. The extent of increase in GSH-Px activity was greater in the HC (91%) than in the CC (66%) of old supplemented trainees. The same trend was seen between the regions i.e. HC exhibiting greater increase than CC in the adult and middle-aged too (Table 1).

Values are mean ± S.E. of four animals per group. Glutathione peroxidase activity is expressed as μmol NADPH oxidized per minute per mg protein. SE-C (N), sedentary controls; SW-T (N), swim trainees; SE-C (+E), sedentary controls supplemented with Vitamin E; SW-T (+E), swim trainees supplemented with Vitamin E; mos, months; HC, hippocampus; CC, cerebral cortex. (a–d) Values with different superscripts between groups in each column are significantly different (P < 0.05).

Table 1

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Values are mean ± S.E. of four animals per group. Superoxide dismutase activity is expressed as μmol NADPH oxidized per minute per mg protein. SE-C (N), sedentary controls; SW-T (N), swim trainees; SE-C (+E), sedentary controls supplemented with Vitamin E; SW-T (+E), swim trainees supplemented with Vitamin E; mos, months; HC, hippocampus; CC, cerebral cortex. (a–d) Values with different superscripts between groups in each column are significantly different (P < 0.05).
higher increase in SOD activity in the HC (63.6% of SE-C, N) than in the CC (46.5% of SE-C, N) \( (P < 0.05) \) (Table 2). A combination of exercise training and Vitamin E in 4-mo-old resulted in significant increase in CAT activity (28% in HC and 48% in CC of SE-C, N) \( (P < 0.05) \). In the 8-mo-old too, significant increase in enzyme activity was seen in both the regions (41% in HC and 59% in CC of SE-C, N). Exercise training or Vitamin E by themselves were beneficial to both the regions in terms of increasing the CAT activity. Middle-aged rats exhibited an increase in the unsupplemented trainees by 46% in HC and 24.4% in CC of SE-C (N). Similarly in the old, a notable feature of this enzyme was that its activity increased significantly only in the unsupplemented trainees (15% in HC and 40.4% in CC of SE-C, N) but not in response to either supplementation or a combination of Vitamin E plus exercise (Table 3).

3.1.1. Lipid peroxidation

3.1.1.1. MDA. MDA as an index of LP decreased in the 4-mo-olds in both the regions although insignificantly between the regions and the extent of reduction was maximal (63% in HC and 78% in CC of SE-C, N) in the supplemented trainees (Fig. 1a). In the 8-mo-old, significant reduction in the MDA was seen between the regions in all the groups. The extent of reduction was maximal in the HC (53.3% of SE-C, N) and CC (30.0% of SE-C, N) of supplemented trainees (Fig. 1b). In the 12-mo-old, reductions were seen only in the HC (27% of SE-C, N), while CC showed increases in MDA. In the CC, MDA was maximal in the unsupplemented trainees supplemented with Vitamin E (Fig. 1c). In contrast to the above ages, 22-mo-olds (Fig. 1d) showed increase in MDA in trainees and this was irrespective of supplementation. However, supplemented sedentary exhibited significant decrease in MDA content in the HC (30% of SE-C, N) as well as CC (9.2% of SE-C, N).

3.1.1.2. LF-like AFS. As indicated in Fig. 2a, exercise training alone was not effective in reducing the fluorescent substances in the 4-mo-olds. However, Vitamin E supplemented sedentary showed significant reductions in the substances when compared to unsupplemented seden-
Fig. 2. Lipofuscin-like autofluorescent substances in the brain in relation to exercise and Vitamin E supplementation in (a) 4-mo-olds, (b) 8-mo-olds, (c) 12-mo-olds and (d) 22-mo-olds. Values are means ± S.E. (n = 4 per group). Significance between group means of four ages is analyzed by DMRT and represented in upper case. Animals of all ages show significant variation between regions. P < 0.05. Those not sharing the same letters are significantly different. HC, hippocampus; CC, cerebral cortex.

4. Discussion

Specific age-related neurodegenerative diseases of the brain such as Alzheimer’s implicate increased vulnerability to OS that generates free radicals [19]. The aging process by itself exemplifies the cumulative effect of deterioration of cells and tissues from free radicals. Although the brain has built-in mechanisms to counteract these radicals, the antioxidant defense is overwhelmed by the aging process besides disease. We have looked in an animal model to see whether different parts of the brain, namely the cerebral cortex and hippocampus respond to exercise training when initiated in different ages that encompass the entire span of life. Our initial studies in rats have demonstrated age-related biochemical adaptations in the CC [3] and a
better endurance capacity to swim training in the Vitamin E supplemented rats [4]. Our approach has also attempted to find an answer to an intriguing problem i.e. Will middle age and old brains respond to only dietary antioxidants or to only exercise training or to a combination of both in terms of improving their antioxidant defenses?

The increase in LP with age in many tissues has been reported to occur concomitantly with increased DNA and protein oxidation, and mitochondrial OS in animal as well as human models[2,27,31]. Our results on statistically insignificantly indicate of enhanced detoxification of H2O2 and LPOs.

Perhaps a treatment of Vitamin E and exercise training may have induced adaptive changes in the antioxidative defenses in order to compensate for greater free radical generation in the CC and HC. Increased CAT activity in response to Vitamin E as well as to exercise in the HC of all age groups although to a lesser extent in the middle-aged and old, suggests the production of excess amount of oxygen from H2O2. Thus, it is likely that the decrease in the CAT and GSH-Px activity in the cerebral cortex and hippocampus of the old may predispose the area to oxidative attack. Decreased CAT activity in the old suggests increased enzyme protein oxidation due to H2O2 accumulation. Our results have indicated that the lower the basal levels of the brain CAT and GSH-Px, the more sensitive was the brain to Vitamin E. Thus, the lowest basal levels of hippocampal GSH-Px in the 22-mo-old sedentary rats induced significantly higher extent of enzyme increase (43.4%) in the Vitamin E supplemented sedentary as compared to the 4- and 8-mo-olds, wherein only a 26 and 21% increase were seen in the SE-C(E) and thereby suggesting that this group is more sensitive to Vitamin E and therefore requiring more of the antioxidant to stimulate them. Our results are somewhat compatible with that reported by Hoessain et al.[12] on the effects of docosahexaenoic acid in the cerebrum for GSH-Px activity. A remarkable feature of the middle-aged was that although the basal levels (27.7 μmol NADPH oxidized per minute per mg) i.e. of SE-C(N) were the same as that of the young adult counterpart, the extent of increase in GSH-Px activity in the HC of the middle-aged was higher (53%) than that seen in the young adult (26%), suggesting that age may be a crucial factor that can enhance the requirements for an antioxidant in this region. Training plus supplement elevated GSH-Px activity rather than just the supplement in the CC of middle-aged and old rats. CAT failed to respond to either Vitamin E or training in the HC as well as CC in the middle-aged and old. A combination of both however induced greater increase in the CC than in the HC in the young and old adults.

Vitamin E seems to offset the age-related decline in basal levels of SOD activity in the HC and CC of supplemented sedentary. However, the extent of increase seen in the HC in response to Vitamin E was significantly higher in the 12-mo-old as compared to the 4- and 8-mo-old. A combination of the supplement and exercise resulted in maximal increase in the old followed by the middle-aged, old adult and young adult. The enhanced activity may explain the possible induction of antioxidant enzyme system to scavenging reactive oxygen species (ROS) that is generated with age and exercise, more so through the latter in the old. Insignificant increase in SOD activity was evident in the cortex of supplemented 4- and 22-mo-olds as analyzed by DMRT. However, exercise plus Vitamin E evoked maximum increases in the 22-mo-old (46%) with 4- and 12-mo-olds showing an increase by 33%.

HC had higher basal SOD activity than the CC in un-supplemented sedentary of all the age groups, with the basal levels of LF-like AFS in this group lower in the HC than in the CC.
the CC. As the levels of this enzyme increased in the SW-T (E), lower levels of LP was evident as seen by the LF-like AFS and it appeared to be harmonized by the modulation of regional GSH-Px activity. Our findings on increased level of LP in terms of MDA and LF-like AFS with age in the two regions are similar to that reported by Ando et al. (1990) and thus perhaps might support the results on increased cholesterol accumulation in the brain tissue [16].

CAT showed higher basal levels in the CC than in the HC, while GSH-Px in these two regions showed higher level in the middle-aged and old but not in the young and old adults. The regional differences that we have seen in terms of both antioxidative enzymes and extent of LP may be speculated in terms of region-specific enzyme activity [26,33], lipid composition [35] and the extent of generation of ROS [21]. In the present study, increase in basal MDA levels both the regions of the old suggests the contribution of ROS in promoting brain aging. Our results are in concordance with that reported on studies using rat and mice brain [17,22]. Although we have not measured ROS, it may be appropriate to mention that in vitro stimulation by iron and H2O2 in the young and old animals result in an age-related decrease in the magnitude of response, which are correlated to alterations in the fatty acid composition during aging. An increase in MDA synthesis following iron and peroxide stimulation has been reported in aged mice [13,28]. Adult mice have better capacity to buffer exogenous stimuli and in contrast to aged mice, which are no longer able to counteract the enhanced production of MDA. In fact, studies on ROS by Leitner et al. [17] using probes DHR 123 and DCF who along with Back et al. [5] found no age-related differences in the ROS production in the hippocampus. These findings indicate that the increased LPO levels in the brain is not due to increased levels of precursor polyene fatty acids, but rather to altered protective systems against LP [2]. Our results on AOsEs also support this view. Further, increase in these enzymes may be a ROS-mediated response along with superoxide anion, which may play significant role as a regulatory mediator in signaling processes to protect the cells from free radical injury [7]. The relationship between the activities of GSH-Px and SOD in the CC and HC suggests that the two enzymes act in concert.

Based on our findings, we propose that low concentration of AOsEs in the cortex and hippocampus with age makes it more vulnerable to OS and enhancing their activities through moderate exercise in combination with Vitamin E might offer a better line of defense against lipid peroxidative stress in both the regions. Vitamin E is reported to protect the mitochondrial membranes of rat brain from the toxic effects of peroxidation [34] through reducing free radicals. Dietary supplements of antioxidants are reported to be beneficial in reducing age-related declines in neuronal signal transduction as well as cognitive deficits in aged rats [14]. Thus, the findings of our present study suggest that an integration of exercise training and Vitamin E may emerge as a useful neuroprotector against age-related decline in the antioxidant enzymes and increased lipid peroxidation. Hippocampus and cerebral cortex which are the sites for memory and learning are benefited from training plus Vitamin E even when initiated as late as middle- and old-age. Although our results are on an animal model, it gives us a rationale to look into this integrated dual method for reversing age-dependent brain dysfunction.

Acknowledgments

The present study was supported in part by a Grant-in-Aid from the Department of Science and Technology, New Delhi, India (No. SP/SOB-32/96 to S. Asha Devi) and the University Grants Commission, New Delhi, India (No. F3-62/98/SR-II, to S. Asha Devi). We wish to thank Ms. S. Prathima for technical assistance and Mr. A.K. Goyal for statistical analyses.

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