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Oxidative Damage of Rat Cerebral Cortex and Hippocampus, and Changes in Antioxidative Defense Systems Caused by Hyperoxia

KOJI ONODERA[a], NAO-OMI OMOI[a], KOJI FUKUI[a], TAKAHIRO HAYASAKA[a], TADASHI SHINKAI[b], SHOZO SUZUKI[b], KOUICHI ABE[c] and SHIRO URANO[a,*]

[a]*Shibaura Institute of Technology, Division of Biological Chemistry, 3-9-14 Shibaura, Minato-ku, Tokyo 108-8548, Japan;* [b]*Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan;* [c]*Vitamin E Information and Technology Section, Eisai Co., Ltd., Tokyo, Japan*

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In order to elucidate the oxidative damage in rat brain caused by oxidative stress, regional changes in the levels of lipid peroxidation products and antioxidative defense systems in cerebral cortex and hippocampus, and in their synapses, which modulate learning and memory functions in the brain, were studied. When rats were subjected to hyperoxia as an oxidative stress, thiobarbituric acid reactive substance (TBARS) in the regions studied increased more than in normal rats by approximately 35%. The values in oxygen-unexposed vitamin E-deficient rats were also higher than in normal rats. It was found that the TBARS contents in synaptosomes isolated from both regions were remarkably higher than in the organs. These results imply that synapses are more susceptible to oxidative stress than the organ itself. This tendency was also observed in the content of conjugated diene. In response to oxidative stress, the status of the antioxidant defense system in each region, i.e. the concentration of vitamin E, and the activities of superoxide dismutase, catalase and glutathione peroxidase, decreased remarkably. On the other hand, in oxygen-unexposed vitamin E-deficient rats, the activities of these enzymes in each region tended to increase, except for catalase activity. These results suggest that in response to the oxidative stress, the antioxidant defense systems may be consumed to prevent oxidative damage, and then, may be supplied through the antioxidant network.

Keywords: Brain; Oxidative damage; Synapse; Cerebral cortex; Hippocampus; Vitamin E

INTRODUCTION

Oxidative stress is known to produce various reactive oxygen species (ROS) in biological systems, including superoxide, hydrogen peroxide, and hydroxyl radicals.[1] It has been recognized that ROS are generated as a consequence of the leakage of electrons from specific segments of the electron transport system in mitochondria, and that ROS may attack organs to induce aging and various disorders, including arteriosclerosis, diabetes, cancer, and senile dementia, due to excessive free radical reactions.[2–6] The major physiological targets of ROS in living tissues are presumed to be polyunsaturated lipids, proteins, and nucleic acids. Attacks on these molecules result in physiological dysfunctions, leading to several disorders derived from oxidative damages.[7,8] Among organs in living systems, the brain is considered to be more susceptible to oxidative stress than other tissues due to its high content of polyunsaturated lipid-rich neural parenchyma, high oxygen utilization accounting for one-fifth of total systemic consumption and low levels of antioxidative enzymes.[9–11] Furthermore, there is considerable evidence that neurotoxic iron accumulates in rat brain during aging, and that an increase in iron in brain through either the liberation from iron-bound ferritin, or an artificial injection of an iron solution into brain induces ROS, resulting in a significant increase in thiobarbituric acid reactive substance (TBARS) and conjugated dienes in brain.[12–15].

Based on these findings, it is speculated that several degenerative changes in brain function may

*Corresponding author. Tel.: +81-3-5476-2429. Fax: +81-3-5476-3162. E-mail: urano@sit.shibaura-it.ac.jp

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be mediated by iron-induced peroxidative processes following dysfunction of the antioxidative defense systems. However, the role of oxidative events in the functional states of brain and their deleterious consequences in the nervous system remains poorly understood.

Previous studies on brain aging have shown a high level of oxidative damage during normal brain aging, as well as in senile dementia.[16–18] Dementia is considered to be an acceleration of normal aging in affected brain regions which undergo progressive damage by ROS.[19] Recently, we reported that when rats are subjected to hyperoxia as an oxidative stress, several morphological changes, such as the swollen mitochondria, deformed nuclei in nerve cells, pigmentation, and an abnormal accumulation of synaptic vesicles in nerve terminals, are observed by electron-microscopy.[16,20] Furthermore, we also found that acetylcholine release from synaptosomes was significantly decreased following stimulation by potassium chloride.[20] These phenomena suggest the presence of deficits in neurotransmission caused by oxidative stress, and may ultimately lead to dementia. The cerebral cortex and hippocampus regions that are thought to control the cognitive and motor functions, seem to rely on several antioxidants for further protection.[21] In fact, a recent report revealed that treatment with vitamin E significantly delays the progression of Alzheimer's disease.[22] The extent of oxidative stress to brain is probably exacerbated by decreased efficiency in the antioxidative defense systems, and by increases in the lipid peroxide status. While there are many reports of changes in the activity of antioxidative enzymes, i.e. superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHPx), etc., during aging, the effects remain controversial due to discrepant results, that is, both an increase and a decrease in the activities of SOD and GSHPx in rat brain during aging have been reported previously.[23–26] The discrepancy is considered to be caused by species and sex differences, experimental conditions and circumstances. In view of the above, according to the free radical theory of brain aging,[31] we have examined hyperoxia-induced changes in lipids and antioxidant status in either the rat cerebral cortex and hippocampus or freshly isolated synaptosomes from corresponding regions.

Although it has been criticized questionably that hyperoxia induces ROS in living tissues, there are several reports that ROS, such as hydrogen peroxide and superoxide are generated by hyperoxia in lung, heart muscle, brain and erythrocytes of several animals,[27–29] and that the rate of ROS generation in lung is in proportion to the concentration of oxygen breathed.[30] Based on these findings, it is reasonable to consider that hyperoxia as an oxidative stress used in this study may induce ROS in rat brain. This idea is also supported by the changes in the activity of antioxidative enzymes and an increase in lipidperoxides through hyperoxia.[16,20]

MATERIALS AND METHODS

Animals

All experiments with animals were performed with the permission of the animal protection and ethics committee of Shibaura Institute of Technology. Male Wistar rats (three months old), were fed a standard diet (α-tocopherol content: 3–5 mg/100 g diet) prior to use. To compare the susceptibility to oxidative stress, vitamin E-deficient rats fed a vitamin E-deficient diet (no tocopherols were detected by HPLC analysis, Funabashi Nojo, Chiba, Japan) for 9 weeks were also used. Animals were maintained under 100% oxygen as an oxidative stress at 20°C for 48 h in the oxygen chambers described previously.[32] In order to compare with an influence of hyperoxia in the oxygen exposed rats, the normal control and vitamin E-deficient rats were maintained under air.

Isolation of Synaptosomes

The rats were sacrificed by decapitation, and the cerebral cortex and hippocampus were quickly separated from the brain and homogenized separately in ice-cold 10 mM Tris–HCl buffer, pH 7.4, containing 0.32 M sucrose and 1 mM EDTA in a Glass/Teflon homogenizer using 12 strokes with a tight-fitting pestle. The homogenates were centrifuged at 3000*g* at 4°C for 3 min. The resulting pellets were re-homogenized and centrifuged as described above. The two supernatant fractions were combined and centrifuged at 20,000*g* for 10 min at 4°C. The resulting pellet was suspended in 6 ml of 12% Ficol solution in Tris–HCl buffer and re-homogenized by 5 strokes of a loose-fitting pestle as described above. The homogenate obtained was centrifuged at 97,000*g* at 4°C for 1 h through three-step discontinuous Ficol density gradients consisting of 12, 7.5 and 0% Ficol. The synaptic fraction obtained was washed with Krebs–Ringer's buffer [KRB; 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.4, 10 mM sucrose, 143 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$], and suspended in a small volume of KRB. The synaptosomes obtained were used immediately for all studies.

TABLE I Effect of oxidative stress on the contents of TBARS, conjugate dienes, and phospholipids in cerebral cortex, hippocampus, and in their synapses isolated from rats

Parameter	Value for Control (20% O_2)	Hyperoxia (100% O_2)	VE deficient (20% O_2)
TBARS (nmol/mg protein)			
Cerebral cortex	0.827 ± 0.012	1.147 ± 0.015*	1.152 ± 0.027**
Synaptosomes	2.383 ± 0.033	4.398 ± 1.303*	4.870 ± 2.027**
Hippocampus	0.724 ± 0.015	0.965 ± 0.134	1.280 ± 0.062*
Synaptosomes	2.569 ± 0.065	4.235 ± 0.121**	3.846 ± 0.061*
Conjugated diene (nmol/mg protein)			
Cerebral cortex	8.740 ± 1.323	12.820 ± 1.354	17.612 ± 2.187*
Synaptosomes	23.740 ± 1.893	29.820 ± 3.533**	27.612 ± 2.061**
Hippocampus	7.932 ± 2.380	11.796 ± 1.846	12.324 ± 2.364*
Synaptosomes	26.081 ± 1.332	34.354 ± 2.476**	30.245 ± 1.835**

Values are means ± SE, $n = 12$ *$p < 0.01$, **$p < 0.05$ versus Control.

Analyses of the Lipids of Cerebral Cortex and Hippocampus and their Synaptosomes

TBARS levels were measured as previously reported by Ohkawa *et al.*[33] The content of TBARS is expressed as nmol equivalents of malondialdehyde per mg protein in the samples. Conjugated dienes, formed by the peroxidation of unsaturated fatty acids, were analyzed as previously reported.[34]

Measurement of the Activity of Antioxidative Enzymes and Vitamin E Content

An aliquot of the homogenate was mixed with 1% NaCl solution (0.5 ml), 6% pyrogallol solution in ethanol (2 ml), 35% KOH solution (1 ml) and 2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (240 pmol) as an internal standard. The mixture was saponified at 100°C for 45 min. After cooling, 1% NaCl solution and a mixture of hexane–ethyl acetate (9:1, by vol.) were added. The extracts were evaporated under nitrogen gas, and methanol (200 μl) was added to the residue. The solution was analyzed by HPLC with an electrochemical detection. The chromatographic conditions used for HPLC analysis were identical to those described earlier.[35] The activity of SOD was analyzed by the reduction method of nitroblue tetrazolium using the analysis kit, SOD Test Wako (Wako Pure Chemical Industries, Osaka, Japan). The activities of catalase and GSHPx were assessed by previous.[36,37]

Statistical Analysis

The results are presented as mean ± SE. All data were assessed by the ANOVA analysis, and a p value less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Lipid Content in Cerebral Cortex, Hippocampus, and their Nerve Terminals

Several reports have indicated that in neurodegenerative disease the affected regions in the brain are subjected to inordinate levels of oxidative stress, and tend to show increased lipid peroxidation, resulting in impairments of cognitive performance such as learning and memory.[38,39] Consequently, in order to confirm whether the impaired cognitive function is related to lipid peroxidation in the brain, we analyzed the changes in lipid contents caused by hyperoxia in the cerebral cortex and hippocampus, and their nerve terminals in rats, as these regions are considered to control learning and memory functions in the brain. As shown in Table I, it was found that the amounts of TBARS in cerebral cortex and hippocampus of rats subjected to hyperoxia were significantly higher than those in oxygen-unexposed control rats by approximately 33–39%. The values in the synaptosomes from the corresponding organs were significantly elevated by oxidative stress. When vitamin E-deficient rats were maintained under normal condition (20% O_2), the TBARS values were also remarkably increased in cerebral cortex and hippocampus, and their synapses. Synaptosomes isolated from each region revealed approximately three- to four-fold differences in TBARS values of the corresponding organs. These TBARS values obtained in this study are compatible with those in the previous reports.[40–42]

Conjugated dienes, which are formed during the peroxidation of polyunsaturated lipids, are another indicator of lipid peroxidation. The levels of conjugated dienes in cerebral cortex and hippocampus, and corresponding synapse were also increased significantly by oxidative stress and vitamin E-deficiency in a way similar to the changes in TBARS (Table I). These results may imply that

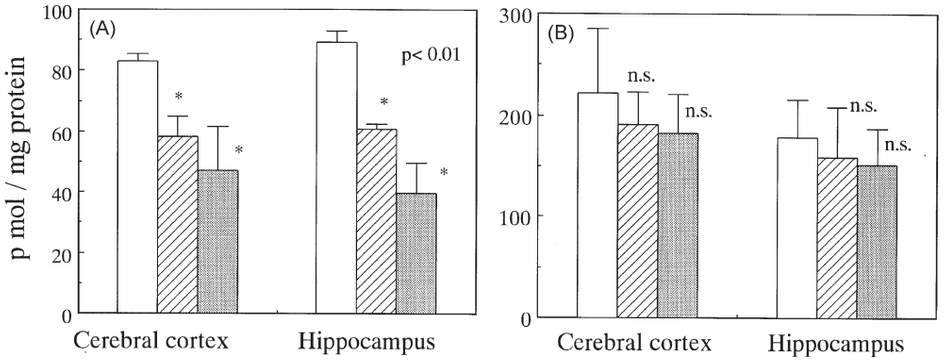

FIGURE 1 Changes in vitamin E content in cerebral cortex, hippocampus (A), and in synaptosomes from the corresponding regions (B). Open bars, rats maintained in air; slashed bars, rats exposed to hyperoxia; dotted bars, vitamin E deficient rats maintained in air. $^*p < 0.01$ versus normal control; n.s., not significant.

synapses in each region are more susceptible to oxidative stress than the organ itself.

It has been recognized that the fusion of synaptic vesicles with synaptic plasma membranes is necessarily needed in the neurotransmission process. Since membrane lipids play an important role in the membrane fusion, an increase in lipidperoxides in synaptic plasma membranes may induce a dysfunction of neurotransmission. In fact, the phenomena of an abnormal accumulation of synaptic vesicles containing neurotransmitter in nerve terminals, a decrease in acetylcholine release from synaptosomes, and a remarkable increase in the TBARS in synaptic plasma membranes caused by hyperoxia were reported previously.[16,20] A dysfunction of neurotransmission may be impaired cognitive function. However, it is difficult at present to define that only lipidperoxides in synapses induce the decline of cognitive function. Other factors, such as oxidized proteins, should be assessed.

On the basis of this consideration and results obtained in this study, it is interesting to presume that the oxidative damage in nerve terminals caused by ROS may induce the deficit in neurotransmission, resulting in a decline of cognitive function.

Changes in Antioxidative Defense Systems Through Oxidative Stress in Cerebral Cortex and Hippocampus, and their Nerve Terminals

One possible explanation for an influence of oxidative stress to living tissues is a perturbation in the levels of antioxidants involved in ROS neutralization. These antioxidants include both enzymatic systems, such as SOD, catalase and GSHPx, and nonenzymatic ones, such as vitamin C and E and glutathione. In general, no antioxidant acts separately against oxidative stress, but, rather, the actions are complimentary within an antioxidative network.[43] To better understand the protective effect of antioxidants against oxidative damage in brain, it is important to assess the changes in enzyme activities during the peroxidation process. In order to confirm whether the oxidative damage to the cerebral cortex and hippocampus, and the corresponding synapses is accompanied by changes in defense systems, the concentration of vitamin E, and the activities of SOD, catalase and GSHPx were analyzed.

As shown in Fig. 1A, it was found that the vitamin E (α-tocopherol) content in cerebral cortex and hippocampus is decreased significantly by hyperoxia. In vitamin E-deficient rats maintained under air, the vitamin E content in each region is about half that in the normal controls. On the other hand, although the content in synaptosomes from each region is somewhat decreased by hyperoxia, the decrease in vitamin E level was not significant (Fig. 1B). The decrease in the vitamin E content in synaptosomes from vitamin E-deficient rats maintained under air conditions was not remarkable. On the basis of these results, it is obvious that synapses are much more susceptible to oxidative stress than the brain tissue itself, and that since the nerve terminals need further vitamin E to protect against oxidative damage in response to the stress, its levels are then transferred from other sites in the brain. A similar tendency was also observed in the activities of antioxidative enzymes (Table II). Although the activities of SOD and GSHPx in the cerebral cortex and hippocampus decreased significantly in response to oxidative stress, in the synapses from the corresponding regions of rat brain, both enzymes were induced by the stress. The catalase activity in each organ and its synapses was decreased by hyperoxia. These results suggest that the catalase activity might decrease without further induction; in contrast, SOD and GSHPx might be transferred from surrounding brain tissue to nerve terminals in order to detoxify ROS, and hence the activities of SOD and GSHPx might be lowered. Thus, it is obvious that since synapses are more

TABLE II Changes in the activities of superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalse in cerebral cortex, hippocampus and in their synapses isolated from rats

Parameter	Value for		
	Control (20% O_2)	Hyperoxia (100% O_2)	VE deficient (20% O_2)
SOD (U/mg protein)			
Cerebral cortex	0.719 ± 0.146	0.353 ± 0.086*	1.338 ± 0.089**
Synaptosomes	6.461 ± 0.512	10.761 ± 0.887**	8.762 ± 0.729
Hippocampus	0.873 ± 0.176	0.320 ± 0.087*	0.879 ± 0.099
Synaptosomes	7.866 ± 1.341	9.563 ± 0.121**	8.846 ± 0.932
Catalase (μ mol/mg protein)			
Cerebral cortex	6.896 ± 1.352	3.857 ± 0.606*	2.820 ± 0.343*
Synaptosomes	6.614 ± 1.053	5.372 ± 0.773	4.612 ± 2.061
Hippocampus	14.320 ± 1.773	7.233 ± 1.171*	4.292 ± 0.770
Synaptosomes	10.329 ± 1.971	6.471 ± 1.181**	7.131 ± 1.035
GSHPx (Δ 340 nm/mg protein)			
Cerebral cortex	0.049 ± 0.008	0.030 ± 0.006**	0.029 ± 0.011**
Synaptosomes	0.039 ± 0.003	0.044 ± 0.014	0.040 ± 0.006
Hippocampus	0.047 ± 0.013	0.030 ± 0.005**	0.031 ± 0.011**
Synaptosomes	0.051 ± 0.025	0.114 ± 0.019*	0.084 ± 0.037

Values are means ± SE, $n = 12$ *$p < 0.01$, **$p < 0.05$ versus Control.

vulnerable to oxidative damage than the brain regions, these enzymes may be induced in response to oxidative stress. Generally, it has been recognized that GSHPx can replace catalase as a scavenger of hydrogen peroxide in the brain because of the low catalase activity in brain.[44] Consequently, it is likely that the GSHPx activity in synapses will increase in order to degrade hydrogen peroxide and will then be induced to detoxify the accumulated lipid hydroperoxides formed in response to the chain breaking reaction of vitamin E.[45] Although surprisingly, it was found that the SOD activity increased remarkably in vitamin E-deficiency, it is difficult to explain why vitamin E-deficiency contributes to an over-induction of SOD through the antioxidant network in the brain. This work was focused on the oxidative damage in cerebral cortex and hippocampus, because these regions are considered to relate to cognitive function in brain. However, we also observed that other areas of brain were also affected by hyperoxia and vitamin E-deficiency (data not shown).

In conclusion, since it is known that neurological abnormalities observed in vitamin E-deficiency are similar to those observed in neurological disorders in the aged, it seems likely that these dysfunctions may be caused by oxygen-derived free radicals.[46,47] This consideration is supported by the findings in this study that damages in cerebral cortex and hippocampus caused by hyperoxia is similar to those observed in vitamin E deficient rats. The accumulation of lipid peroxides and changes in antioxidant activity in nerve terminals observed in this study suggest that oxidative stress may be involved in the mechanism of neuronal death, leading to a decline in cognitive functions. Although all of these alterations through oxidative stress may cause a pathological disruption of brain function, it is not easy to fit these findings into the hypothesis that once nerve terminals are peroxidized by ROS, neurotransmission becomes impaired, resulting in the decline in the learning and memory functions observed in senile dementia. However, recently we found that when rats are subjected to oxidative stress, they lose their learning and memory functions remarkably in the water-maze test.[48] Our present findings strongly support this hypothesis. Further studies, however, are needed to elucidate the contribution of oxidative stress to the impairment in cognitive function that occur in aging and dementia.

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