Vitamin E Prevents Hyperoxia-Induced Loss of Soluble N-Ethylmaleimide-Sensitive Fusion Protein Attachment Protein Receptor Proteins in the Rat Neuronal Cytoplasm

Nozomi Kaneai, a Koji Fukui, b Taisuke Koike, c and Shiro Urano* a

a Life Support Technology Research Center, Shibaura Institute of Technology; b Department of Bioscience & Engineering, Shibaura Institute of Technology; 307 Fukasaku, Minuma-ku, Saitama-shi, Saitama 337–8570, Japan; and c Eisai Food & Chemical Co., Ltd.; 2–13–10 Nihonbashi, Chuo-ku, Tokyo 103–0027, Japan.

Received February 18, 2013; accepted July 1, 2013

This study examines the ability of vitamin E to inhibit hyperoxia-induced loss of soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins in the neuronal cytoplasm. Here, the effects of vitamin E on hyperoxia-induced changes in the expressions of N-ethylmaleimide-sensitive factor (NSF) and soluble NSF-attachment protein α (α-SNAP) in the rat brain were analyzed. When rats were subjected to hyperoxia, the expression of both SNARE proteins was markedly decreased compared to normal rats. Vitamin E significantly inhibited the decrease in the expression of NSF in rats subjected to hyperoxia. Rats showed the tendency to improve the loss of α-SNAP by vitamin E-supplementation, although it was not statistically significant. On the other hand, vitamin E deficient rats showed marked loss of these proteins in the brain in the absence of oxidative stress. These results suggest that hyperoxia induces a loss of SNARE proteins, which are involved in membrane docking between synaptic vesicles and presynaptic membranes, and that vitamin E prevents the oxidative damage of SNARE proteins. Consequently, it is implied that vitamin E inhibits impaired neurotransmission caused by oxidative stress through the prevention of oxidative damage to SNARE proteins by probably its antioxidant effect.

Key words neurotransmission; neurodegeneration; vitamin E; N-ethylmaleimide-sensitive fusion protein attachment protein receptor; membrane fusion

It is well recognized that neurodegenerative diseases, such as Alzheimer’s disease (AD), are characterized by the progressive impairment of cognitive function, leading to severe dementia. Since there are increased regional levels of oxidative stress in the brains of patients with neurodegenerative disease, it is reasonable to expect that cognitive deficits may arise from dysfunction in neurotransmission caused by oxidative stress. Oxidative stress occurs in living tissues during periods of imbalance between reactive oxygen species (ROS) generation and detoxification by antioxidants. Consistent with the oxidative stress theory of neurodegeneration, our previous study revealed that rats subjected to hyperoxia exhibit several kinds of neuronal damage in the brain; specifically, swollen mitochondria, pigmentation, deformed nuclei, and the abnormal accumulation of neurotransmitter-enclosed synaptic vesicles. Furthermore, it was found that the release of acetylcholine from nerve terminals markedly decreased. In accordance with these phenomena, young rats subjected to oxidative stress showed marked cognitive impairment.

These results suggest that ROS, generated by hyperoxia, attack the nervous system and induce neuronal cell damage, thereby impairing neurotransmission in the brain at the level of nerve terminals. As mentioned earlier, hyperoxia has been observed to result in an abnormal accumulation of synaptic vesicle in nerve terminals and the cause of this buildup is not well understood. However, it is likely that the process of synaptic vesicle fusion with the presynaptic plasma membrane during neurotransmission may be impaired as a result of hyperoxia, thereby decreasing the release of acetylcholine from nerve terminals. In the fusion process of synaptic plasma membranes and synaptic vesicles, particular proteins, such as the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins, which dock both membranes prior to membrane fusion by lipid-mixing, are important to neurotransmission. The SNARE proteins are localized in pre-synaptic plasma membranes, synaptic vesicles and in the neuronal cytoplasm. It is thought that the docking of synaptic vesicles with pre-synaptic plasma membranes occurs as a result of the formation of a complex composed of synaptic vesicle-associated membrane proteins (synaptobrevin and synaptotagmin), pre-synaptic plasma membrane proteins (synaptosomal associated protein of 25 KDa (SNAP-25) and syntaxin-1), and soluble proteins in the cytosol (N-ethylmaleimide-sensitive factor (NSF) and soluble NSF-attachment protein α (α-SNAP)). Consequently, it is presumed that once these proteins are damaged through oxidative stress, the membrane docking may decline, leading to impairment of neurotransmission. Our recent report revealed that hyperoxia induced a marked reduction in the levels of syntaxin-1, SNAP-25, synaptobrevin and synaptotagmin in the rat brain, and that vitamin E inhibited the hyperoxia-induced denaturation of these proteins. Based on these findings, it is easy to consider that the expressions of NSF and α-SNAP in the cytoplasm may be changed through hyperoxia, and that vitamin E may prevent the denaturation of both SNARE proteins. However, in order to well-understand the influence of hyperoxia on the dysfunction of the neurotransmission as a whole, it is important to assess whether both SNARE proteins in the neuronal cytoplasm are also damaged by hyperoxia, and whether vitamin E prevents the disruption of the membrane docking process caused by hyperoxia.

Since it has been known that hyperoxia induces decreases in antioxidants, and generates ROS in living tissues resulting in the production of lipid peroxides and oxidized proteins, it...
is reasonable to imply that the induction of oxidative stress is caused by hyperoxia. Consequently, we considered that hyperoxia is correlated with oxidative stress in this study.

MATERIALS AND METHODS

Animals and Chemicals All animal experiments were performed with the permission of the Animal Protection and Ethics Committee of the Shibaura Institute of Technology. According to our previous report that vitamin E prevents cognitive deficit caused by hyperoxia,4–6 4-week-old male Wistar rats were fed ad libitum with a standard diet (α-tocopherol content: 3–5 mg/100 g diet) or a vitamin E-supplemented diet (RRR-α-tocopherol, 250 mg/100 g diet) for 9 weeks from 4 weeks of age, followed by exposure to 100% oxygen at 20°C for 48 h in an oxygen chamber. Vitamin E-deficient rats were fed a vitamin E-deficient diet (Funakoshi Nojyo, Chiba, Japan) for 9 weeks from 4 weeks of age. All chemicals were of the highest grade available.

Antibodies Rabbit polyclonal anti-NSF and Mouse monoclonal [4E4] anti-α-SNAP were purchased from Abcam Co., Ltd. (Tokyo, Japan). The immunoglobulin G (IgG) fraction of a polyclonal rabbit antisera to β-galactosidase was obtained from Nordic Immunological Laboratories, Inc. ( Tilburg, Netherlands). Horseradish peroxidase (HRP)-conjugated goat IgG fraction against mouse IgG and HRP-conjugated donkey anti-rabbit IgG were purchased from Promega Corporation (Madison, WI, U.S.A.).

Isolation of Synaptosomes Synaptosomes were isolated from whole brain of rats according to the procedure of Dodd et al.7 using an ultracentrifugation at 150000 × g on 3-step discontinuous Ficoll density gradients.

Immunoblotting Analysis of NSF and α-SNAP An aliquot of the synaptosomal suspension (protein content: 4 µg) was homogenized three times in an ice bath for 5 s using an ultrasonic homogenizer. The homogenate was dissolved into 5 µL of a 3-(3-cholamidopropyl)dimethylammonio)propanesulphonate (CHAPS) cell extract buffer (100 mM PIPES, 4 mM ethylenediaminetetraacetic acid (EDTA), 0.2% (w/v) CHAPS, 110 mM dithiothreitol (DTT), 40 µg/mL leupeptin, 20 µg/mL pepstatin-A, 20 µg/mL aprotinin, 2 mM phenylmethanesulfonyl fluoride (PMSF), pH 6.8) and mixed with a solution of β-galactosidase (1 µL, 500 µg/mL in phosphate buffered saline (PBS)). After the mixture was incubated for 20 min in an ice bath, 125 mM Tris-HCl buffer (pH 6.8), containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.01% (w/v) bromophenol blue and 0.55% (w/v) mercaptoethanol, was added. The mixture was fractionated using 10% SDS-polyacrylamide gel electrophoresis (PAGE) together with a protein molecular weight marker (NIPPON Genetics Co., Ltd., Tokyo, Japan). Separated proteins were transferred onto Immobilon transfer membranes (Millipore, Bedford, MA, U.S.A.). The membranes were washed twice with a 25 mM Tris buffer, containing 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20 (TBS-T, pH 7.4). After blocking non-specific binding sites for 1 h with blocking solution (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween-20 and 2% bovine serum albumin (BSA), pH 7.4), blots were incubated with anti-NSF (1:5000), anti-SNAP (1:500), and anti-β-galactosidase antibodies (1:1000) in Can Get Signal solution 1 at 4°C for 12 h. Blots were washed with TBS-T, and incubated with horseradish (HRP)-linked anti-mouse secondary antibody (1:2000) in Can Get Signal solution 2 for α-SNAP antibody, or an anti-rabbit antibody for all other antibodies. Each HRP-labeled antibody was detected with the enhanced chemiluminescence detection system using aluminoo- analyzer (Las-3000, FUJIFILM Imaging Co., Ltd., Tokyo, Japan). β-Galactosidases was used as an internal standard.

Statistical Analysis Results are presented as means±S.E. Comparisons between multiple groups were made by ANOVA, followed by a Tukey's test. A p-value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

According to the SNARE hypothesis of membrane fusion, NSF and α-SNAP, which are localized in the synaptic cytoplasm, fulfill their functions in docking synaptic vesicles with the pre-synaptic plasma membrane through the formation of

![Figure 1](image1.png)  
**Fig. 1.** Effects of Vitamin E on Changes in NSF Expression Caused by Oxidative Stress (A), and Immunodetection of NSF Expression (B)  
a, control rats; b, rats subjected to oxidative stress; c, rats fed a vitamin E-supplemented diet; d, rats fed a vitamin E-supplemented diet and subjected to oxidative stress; e, vitamin E-deficient rats. * p<0.05 versus control, ** p<0.05 versus rats subjected to hyperoxia; n=6 for each group. Band intensity is expressed as relative to β-galactosidase (internal standard) and assessed using a luminoanalyzer.

![Figure 2](image2.png)  
**Fig. 2.** Effect of Vitamin E on Changes in α-SNAP Expression Caused by Oxidative Stress (A), and Immunodetection of α-SNAP Expression (B)  
a, control rats; b, rats subjected to oxidative stress; c, rats fed a vitamin E-supplemented diet; d, rats fed a vitamin E-supplemented diet and subjected to oxidative stress; e, vitamin E-deficient rats. * p<0.05, versus control, ** p<0.05 versus rats subjected to hyperoxia; n=6 for each group. Band intensity is expressed as relative β-galactosidase (internal standard) and assessed.

![Image](image3.png)
a complex consisting of both SNARE proteins. Although our recent results showed that hyperoxia markedly decreased the levels of other SNARE proteins (synaptobrevin and syntaptotagmin in synaptic vesicles, and syntaxin-1 and SNAP-25 in pre-synaptic plasma membranes,6) the effects of hyperoxia on the expression of NSF and α-SNAP in synaptic cytoplasm have not been examined.

When rats were subjected to hyperoxia, a marked decrease in the expression of NSF and α-SNAP in the brain was observed (Figs. 1, 2). In control animals, hyperoxia decreased NSF and α-SNAP levels, versus normal control, by 30.7% and 40.2%, respectively. A similar tendency was observed in vitamin E deficient rats in the absence of oxidative stress. These results suggest that both SNARE proteins in the rat brain were oxidatively damaged by ROS generated through oxidative stress, similar to the oxidative damage observed in SNARE proteins located in synaptic vesicles and pre-synaptic plasma membranes.6) In fact, it is recognized that oxidative stress is elevated in the brain of AD and aged rats,2,8) and that oxidative damage to SNARE proteins is also found in AD patients and aged rats.9,10) Consequently, it is likely that oxidative stress damages SNARE proteins in the nerve terminal, leading to dysfunctional neurotransmission.

In order to confirm this hypothesis, we fed a vitamin E-supplemented diet to rats before subjecting them to oxidative stress. As shown in Figs. 1, 2, vitamin E markedly prevented loss of NSF following hyperoxia in the cytoplasm. The hyperoxia-induced decrease in α-SNAP expression was not inhibited by vitamin E, although rats showed a tendency to increase in α-SNAP level by vitamin E-supplementation. Since it is impossible to elucidate this phenomenon in the present study, further studies are necessary to define this inconsistency. These results suggest that vitamin E may inhibit damages to SNARE proteins in the synaptic cytoplasm caused by hyperoxia and thereby maintain normal neurotransmission.

Thus, hyperoxia deteriorates the process of docking synaptic vesicles with pre-synaptic membranes during neurotransmission by the induction of oxidative damage to all SNARE proteins in the nerve terminal. Furthermore, based on the results of this study, it is implied that dysfunction of membrane docking through oxidative damage to SNARE proteins contributes to the abnormal accumulation of synaptic vesicles in nerve terminals caused by oxidative stress, as previously reported.3) In this study, we used hyperoxia to assess damages of SNARE proteins.

It is obvious that vitamin E inhibits the dysfunctional neurotransmission caused by oxidative stress, probably through its antioxidant property. The results obtained here may, to a certain extent, explain the fact that vitamin E delays institutionalization, as well as the onset of severe dementia in AD.11)

Acknowledgments This study has been supported, in part, by MEXT-Supported Program for the Strategic Research Foundation at Private University, 2011 and a Grant-in-Aid from Eisai Food & Chemical Co., Ltd.

REFERENCES

1) Katzman R. Alzheimer's disease. N. Engl. J. Med., 314, 964–973 (1986).

2) Keller JN, Schmitt FA, Scheff SW, Ding Q, Chen Q, Butterfield DA, Markesbery WR. Evidence of increased oxidative damage in subject with mild cognitive impairment. Neurology, 64, 1152–1156 (2005).

3) Urano S, Asai Y, Makabe S, Matsu M, Izumiyama N, Ohtsubo K, Endo T. Oxidative injury of synapse and alteration of antioxidative defense systems in rats, and its prevention by vitamin E. Eur. J. Biochem., 245, 64–70 (1997).

4) Fukui K, Ono NO, Hayasaka T, Shinnkai T, Suzuki S, Abe K, Urano S. Cognitive impairment of rats caused by oxidative stress and aging, and its prevention by vitamin E. Ann. N. Y. Acad. Sci., 959, 275–284 (2002).

5) Lin RC, Sheller RH. Structural organization of the synaptic exocytosis core complex. Neuron, 19, 1087–1094 (1997).

6) Kaneai M, Arai M, Takatsu H, Fukui K, Urano S. Vitamin E inhibits oxidative stress-induced denaturation of nerve terminal proteins involved in neurotransmission. J. Alzheimers Dis., 28, 183–189 (2012).

7) Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK, Delaunoy JP. A rapid method for preparing synaptosomes: Comparison with alternative procedures. Brain Res., 226, 107–118 (1981).

8) Urano S, Sato Y, Otonari T, Makabe S, Suzuki S, Ogata M, Endo T. Aging and oxidative stress in neurodegeneration. Biofactors, 7, 103–112 (1998).

9) Shimohama S, Kamiya S, Taniguchi T, Akagawa K, Kimura J. Differential involvement of synaptic vesicle and pre-synaptic plasma membrane proteins in Alzheimer’s disease. Biochem. Biophys. Res. Commun., 236, 239–242 (1997).

10) Shimohama S, Fujimoto S, Sumida Y, Akagawa K, Shirao T, Matsuoka Y, Taniguchi T. Differential expression of rat brain synaptic proteins in development and aging. Biochem. Biophys. Res. Commun., 251, 394–398 (1998).

11) Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, Woodbury P, Growdon J, Cotman CW, Pfeiffer E, Schneider LS, Thal LJ. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer’s disease. N. Engl. J. Med., 336, 1216–1222 (1997).