Neurodegenerative diseases such as senile dementia including Alzheimer’s disease have been characterized by progressive deterioration of cognitive function (1). Previous studies on brain aging revealed the high level of oxidative damage in the brain during normal aging, as well as in dementia (2–4). Dementia is considered to be an acceleration of normal aging in affected brain regions which undergo progressive damage from reactive oxygen species (ROS) (5). It is, therefore, reasonable to assume that the deficit in neurotransmission is caused by oxidative damage to nerve terminals through chronic oxidative stress experienced over a long time, resulting in a cognitive dysfunction. In terms of neurodegeneration, the amount of oxidative damage to synapses in the brain regions, which modulate cognitive and motor functions, seems to depend on the protection afforded by several antioxidants (6). Accordingly, it seems that long-term vitamin E supplementation may prevent the oxidative damage of the nervous system during aging. In fact, a previous report revealed that long-term high-dose supplementation of vitamin E to aged individuals provides significant enhancement in cognitive function (7). A clinical trial on vitamin E supplementation in patients with moderately severe Alzheimer’s disease showed delays in institutionalization and the onset of severe dementia (8).

Our previous findings revealed that rats subjected to hyperoxia as oxidative stress show several kinds of neural damage in the brain, that is, swollen mitochondria, deformed nuclei, pigmentation and a quite abnormal accumulation of synaptic vesicles containing neurotransmitters in nerve terminals, and that the release of acetylcholine from synaptosomes was markedly decreased following the stimulation by potassium chloride (4, 9). Furthermore, lipids in pre-synaptic plasma membranes were peroxidiized markedly by ox-

Influence of Oxidative Stress on Fusion of Pre-Synaptic Plasma Membranes of the Rat Brain with Phosphatidyl Choline Liposomes, and Protective Effect of Vitamin E

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Summary Influence of oxidative stress on fusion of pre-synaptic plasma membranes with phosphatidylcholine (PC) liposomes as a model of synaptic vesicle was investigated. The inhibitory effect of vitamin E on the decline in the fusion caused by oxidative stress was also assessed. Rats subjected to hyperoxia as oxidative stress showed significant increases in the levels of lipid hydroperoxides and protein carbonyl moieties in pre-synaptic plasma membranes in the brain. The ε potential of pre-synaptic membrane surface was decreased markedly. When synaptosomes were incubated with PC liposomes labeled by either rhodamine B or calcein as a fluorescence probe, or 12-doxyl stearic acid as an ESR spin trapping agent, translocation of each probe into oxidatively damaged pre-synaptic membranes was decreased significantly. Fatty acid composition analysis in pre-synaptic membranes obtained from normal rats revealed a marked increase in linoleic acid and a moderate decrease in docosahexaenoic content after the incubation. Such changes caused by hyperoxia were inhibited by vitamin E treatment of rats. These results suggest that oxidative damage of pre-synaptic membranes caused by oxidative stress lowers the lipid-mixing for the membrane fusion. The results of this study imply that vitamin E prevents the deficit in neurotransmission at nerve terminals due to the decline in fusion between pre-synaptic membrane and synaptic vesicles caused by oxidative membrane damage.

Key Words vitamin E, neurotransmission, synaptic membrane fusion, oxidative damage, lipid-mixing

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dative stress (9). These phenomena imply that the fusion of synaptic vesicles with pre-synaptic plasma membranes for neurotransmission is lowered due to the oxidative damage to synapses, and hence, synaptic vesicles are abnormally accumulated in nerve terminals. Therefore, it is interesting to assume that these abnormalities in neurotransmission induce the cognitive dysfunction. In fact, we observed that when rats were subjected to oxidative stress, their learning and memory functions markedly declined (10, 11). It has been recognized that the chemical neurotransmission at nerve terminals occurs through exocytotic release of neurotransmitter by regulated fusion between pre-synaptic plasma membrane and synaptic vesicles which store the transmitter. In response to chemical stimuli, calcium ions flow into the pre-synaptic area through the voltage-dependent calcium channels (12). Increased calcium ions in pre-synaptic space involve the reaction of specific proteins for the docking of synaptic vesicles to pre-synaptic plasma membranes before the lipid-mixing membrane fusion, that is, the binding of SNARE protein (synaptobrevin) in synaptic vesicles to SNARE proteins (SNAP-25 and syntaxin) in pre-synaptic membranes (13). However, this theory explaining the mechanism of membrane fusion for chemical neurotransmission is still unclear. It has been known that the lipid component of membranes acts as a regulator of important physiological membrane functions as well as an essential element for cell fusion. On the basis of these theories, it is presumed that once proteins and lipids in pre-synaptic membranes are oxidatively damaged by ROS, the chemical neurotransmission in nervous systems would decline. Although there are many reports related to membrane fusion (14, 15), no influence of the oxidative damage of synaptic plasma membrane caused by an oxidative stress on membrane fusion for neurotransmission has been reported. The present study aimed to determine whether or not the oxidatively damaged pre-synaptic plasma membranes fuse with phosphatidylcholine (PC) liposomal membranes as a model of synaptic vesicles by the lipid-mixing, and whether vitamin E can protect against a deficit in the membrane fusion.

MATERIALS AND METHODS

Animals. All animal experiments were performed with the permission of the Animal Protection and Ethics Committee of the Shibaura Institute of Technology. Male Wistar rats (3 mo old) were fed ad libitum with a standard diet (a-tocopherol content: 3–5 mg/100 g diet), or vitamin E-supplemented diet (250 mg/100 g diet) for 9 wk from 4 wk old, followed by exposure to 100% oxygen at 20°C for 48 h in the oxygen chamber. To compare the influence of oxidative stress, rats were fed with a vitamin E-free diet (no tocopherols were detected by HPLC analysis, Funabashi Nojo, Funabashi, Japan) for 9 wk from 4 wk old and they were kept in ordinary air at 20°C for 48 h prior to experiments. Nine rats were used in each group for each experiment.

Chemicals. Rhodamine B (R18) and Ficoll were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Calcein was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 12-Doxyl stearic acid (12-DSA) was obtained from Molecular Probe Inc. (Oregon, U.S.A.). RRR-a-Tocopherol was supplied by Eisai Co. Ltd. (Tokyo, Japan). PC was extracted and isolated from egg yolk using a mixture of chloroform and methanol (2:1 by vol) as reported previously (16). All other chemicals were of the highest grade available.

Antibody. Mouse anti-rat syntaxin, family I, subclass: IgG 20, monoclonal antibody was purchased from Wako Pure Chemical Industries, Ltd. and horse anti-mouse IgG biotinylated antibody was obtained from Vector Laboratories Inc. (California) as a secondary antibody.

Preparation of the R18-labeled and the calcein-enclosed single bilayer liposomes. For fluorescent and ESR experiments, the probe-labeled PC liposomes were prepared as follows: For preparation of R18-labeled liposomes, PC (3.3 mol%) and R18 (0.1 mol%) were dissolved in 1 mL of ethanol. According to the method of Batzri and Koran (17), a 100 μL aliquot of the solution was injected into phosphate buffered saline (PBS, pH 7.4, 2.5 mL) through a Hamilton syringe at 37°C to obtain a bilayer liposomal suspension. To prepare a suspension of calcein-enclosed liposomes, 3.3 mol% of PC was dissolved in 1 mL of ethanol, and a 400 μL aliquot of the solution was injected into a 10 μM calcein solution in PBS (pH 7.4, 10 mL). Each suspension was concentrated by an ultra filtration, and purified by a gel filtration on Sephadex G-200 to yield the R18-labeled PC liposomes and the calcein-enclosed liposomes, respectively. The 12-DSA-labeled liposomes were prepared in a similar manner to the preparation of R18-labeled liposomes using an ethanol solution of PC (3.3 mol%) and 12-DSA (13 μmol). Using an ethanol solution of RRR-a-tocopherol (0.17 μmol), R18 (0.1 μmol) and PC (3.3 mol%), liposomes containing a-tocopherol were prepared in a similar manner to the preparation of R18-labeled liposomes. The liposomes obtained were also used for the fusion experiment.

Isolation of synaptosomes. Synaptosomes were isolated from whole brain according to the procedure of Dodd et al. with minor modifications (18). After rats were sacrificed by decapitation, their brains were immediately removed, and were homogenized in an 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 homogenate was centrifuged at 3,000×g at 4°C for 3 min. The resulting pellet was re-homogenized and centrifuged as described above. Two supernatant fractions were combined and centrifuged at 20,000×g at 4°C for 10 min. The pellet was suspended in 6 mL of a 1% Ficoll solution in Tris-HCl buffer (pH 7.4) and re-homogenized by 5 strokes of a loose-fitting pestle as described above. The homogenate was centrifuged at 97,000×g at 4°C for 1 h on 3-step discontinuous Ficoll density gradients consisting of 12, 7.5 and 0% Ficoll. The synaptic fraction obtained was washed with PBS (pH 7.4), and centrifuged at 7,000×g at 4°C for 10 min. We used this
washed with water three times. The hexane solution of chloroform/methanol (2:1 by vol) was mixed with some or lipid in synaptosomes extracted by a mixture of PC-lipo- somes or lipid in synaptosomes immediately after isolation without a burst (○) was diluted with PBS (pH 7.4). Samples were analyzed by the ELISA method.

Fig. 1 Immunostaining of “inside-out” pre-synaptic plasma membranes using the syntaxin antibody. Each suspension of “inside-out” membrane vesicles after a burst (●) and synaptosomes immediately after isolation without a burst (○) was diluted with PBS (pH 7.4). Samples were analyzed by the ELISA method.

synaptosomal fraction for further experiments without an assessment of the purity of synaptosomes because previous report revealed that this fraction includes almost pure synaptosomes (18). According to the previous report (15), the “inside-out” vesicles were prepared using the synaptic fraction obtained. The pellets were mixed with 3 mM phosphate buffer (pH 8.5) to burst synaptosomes, resulting in the “inside-out” membrane vesicles of synaptosomes. The “inside-out” form of the membrane was confirmed by an immunochemical staining using a syntaxin antibody, because syntaxin, which is an essential protein for membrane fusion of synaptic plasma membranes with synaptic vesicles, is located at the inner face of synaptic plasma membranes. As shown in Fig. 1, the “inside-out” membrane vesicles were contaminated in the synaptic fraction in PBS. This result suggests that even when synaptosomes were isolated by the method described above, synaptosomes were partly changed to the “inside-out” form in PBS (pH 7.4). Although it is impossible at present to know the ratio of the “inside-out” vesicle content to that of the “right side-out” vesicles in the suspension, it seems to be reasonable to use the suspension obtained in this study for an assessment of membrane fusion, because the membranes in the suspension were much stained by the antibody. To assess the extent of membrane fusion of the “inside-out” membrane vesicles, the fusion experiment of right side-out membranes was carried out using the synaptic fraction including a bit of the “inside-out” membrane vesicles as a control experiment.

Analyses of synaptic and liposomal membrane lipids.

For analysis of fatty acid composition in pre-synaptic and liposomal membranes, each portion of PC-liposomes or lipid in synaptosomes extracted by a mixture of chloroform/methanol (2:1 by vol) was mixed with butylated hydroxyl toluene. The mixture was heated at 100˚C for 1 h with a 5% HCl solution in methanol. Each reaction mixture was extracted by hexane, and washed with water three times. The hexane solution was evaporated under N₂ gas, and the residue was taken up to 200 µL of hexane. The esterified fatty acids obtained were analyzed by a gas chromatography as previously reported (19). Content of lipid hydroperoxides (LOOH) in pre-synaptic plasma membranes was analyzed as follows: One milliliter of rat brain homogenate was extracted with a mixture of chloroform and methanol (2:1 v/v, 2 mL), and evaporated by N₂ gas. The residue was dissolved by 200 µL of methanol, and an 80 µL aliquot of the solution was mixed with a chemiluminescent solution (a mixture of 0.18 mg isoluminol/mL and 1 mg microperoxidase/mL of 70% methanol, 100:1, v/v). Chemiluminescence of the solution was analyzed using a Luminescence PSN apparatus (Atto Co., Tokyo, Japan) at room temperature according to the reported method (20, 21).

Fusion experiments of synaptic plasma membranes with the probe-labeled and the probe-enclosed liposomes. Each suspension of liposomes (50 µL) was mixed with a suspension of “inside-out” membrane vesicles of synaptosomes in PBS (pH 7.4, 1 mL), and the mixtures were incubated at 37˚C for 30 min. Each mixture was centrifuged for 30 min at 4˚C for 15 min. The precipitate obtained was washed 5 times with PBS (pH 7.4) using centrifugation (6,000×g, 4˚C). After each centrifugation, the supernatant was analyzed to assess the existence of phospholipids in liposomes using a phospholipid analysis kit, phospholipids C-HA test Wako (Wako Pure Chemical Industries, Ltd.). After the fourth washing with PBS, no phospholipids were detected. Although the sizes of the synaptosome and liposome used in this study were not assessed, according to previous reports (22, 23), the diameters of synaptosomes and liposome obtained using these methods are known to be 500–700 nm and 30–110 nm, respectively. Each suspension in PBS (pH 7.4) of pre-synaptic membrane vesicles obtained was purified by a gel filtration on Sephadex G-200. The fluorescence of R₁₈ or calcein in each vesicle was measured at ex. 552 nm, em. 574 nm for R₁₈ and at ex. 489 nm, em. 512 nm for calcein. The ESR signal of 12-DSA in each sample was measured in a flat (quartz) cell at room temperature using ESR spectroscopy (JEOL, JES RE1X for X-band 100 kHz modulation frequency, Tokyo, Japan); instrument setting: center field: 335.0±5.0 mT; microwave power: 8 mW; modulation amplitude, 0.1 mT; gain: 630; time constant, 0.1 s, and scanning time 4 min. Manganese oxide was used as an external standard because it provided a constant signal to which all peak heights were compared. Sample peak height was divided by manganese oxide peak height to give the relative peak height.
RESULTS

Oxidative damage to nerve terminal membranes caused by oxidative stress

To elucidate whether oxidative stress induces oxidative damage in nerve terminals of the brain, the level of LOOH in pre-synaptic plasma membranes from rat brain was assessed. LOOH was used as one of the indices of lipid peroxidation. As shown in Fig. 2A, when rats were subjected to hyperoxia as oxidative stress, the content of LOOH in pre-synaptic membranes was increased by a factor of 1.77. In order to ascertain whether such an increase in LOOH was induced by ROS, the involvement of vitamin E as a radical scavenger against lipid peroxidation was assessed. The LOOH content in pre-synaptic membranes from vitamin E-deficient rats kept in ordinary air was also increased. On the other hand, vitamin E-supplemented rats kept in ordinary air showed more decrease in the LOOH content than the normal control rats. When the vitamin E-supplemented rats were subjected to hyperoxia, the value was about half of that of the control rats subjected to hyperoxia.

In association with lipid peroxidation in pre-synaptic membranes, the level of protein oxidation in pre-synaptic membranes ought to be analyzed for an assessment of the oxidative membrane damage. Usually, the level of protein carbonyl moiety is one of the indices for analysis of protein oxidation. As shown in Fig. 2B, it was found that native proteins in pre-synaptic plasma membranes of the rat brain were oxidatively modified with many carbonyl groups due to hyperoxia. The level of carbonyl moiety in pre-synaptic membranes from vitamin E-deficient rats kept in ordinary air significantly increased as compared with that of control rats, since in vitamin E deficiency, the living body is susceptible to continuous oxidative stress in normal atmosphere, resulting in extensive oxidative damage. However, the vitamin E-supplemented rats did not reveal a marked increase in protein oxidation with or without hyperoxia. Since it is known that the depolarization of pre-synaptic membrane surface is necessary for neurotransmission, it is reasonable to assume that such increases in LOOH and oxidatively modified protein in nerve terminal membranes induce a decrease in the depolarization process.

Fusion of nerve terminal membranes and the probe-labeled liposomes

To determine whether the membrane fusion is lowered due to such oxidatively damaged membranes caused by hyperoxia, the synaptic “inside-out” pre-synaptic plasma membranes were incubated with the probe-labeled liposomes as a model of the membrane fusion in a neurotransmission. When R18 was incorporated in liposomal membranes, the fluorescence intensity of R18 in liposomal membranes was 1.619/100 μL.
liposome suspension. After incubation with “inside-out” synaptosomes, the fluorescence of the probe in synaptic plasma membranes was observed by the intensity of 1.181/mg protein. These results suggest that both the membrane vesicles were fused during incubation, and hence, the probe R18 was translocated into pre-synaptic plasma membranes accommodating other lipids in liposomes by the lipid-mixing. As shown in Fig. 4, it was found that translocation of R18 into synaptosomal membranes from rats subjected to hyperoxia was decreased significantly by about 41%. Although vitamin E-deficient rats kept in ordinary air showed also a decrease in the fluorescence intensity of the probe in synaptosomes, the intensity of the probe did not change with the vitamin E-supplementation even after they were subjected to hyperoxia. In order to determine whether an inhibition of deficit of membrane fusion caused by hyperoxia is a result from antioxidation or promotion of lipid affinity by vitamin E, oxidatively damaged pre-synaptic membrane vesicles were incubated with \( \alpha \)-tocopherol-included liposomes. The fluorescence intensity of the R18 in the damaged synaptosomes was decreased by about 38% (intensity: 0.73/mg protein) after incubation. This result suggests that vitamin E in pre-synaptic membranes inhibits a deficit of membrane fusion caused by hyperoxia due to its antioxidant effect. Although R18 is known to be translocated into the membrane layer, the water soluble calcein moves into the vesicle core from the inside liposomal core. In order to assess that results obtained from R18 experiments were not based on the adhesion of liposomal membrane surface, another probe, calcein, was used in this study. When synaptic membranes were fused by liposomes without the adhesion, calcein translocated should be detected inside synaptic membrane vesicles. The fluorescence intensity of the calcein was observed by 2.48/mg protein after a burst of pre-synaptic membrane vesicles (normal control) incubated with calcein-enclosed liposomes, although little fluorescence was observed before the burst (0.26/mg protein). The incorporation ratio of the probe into normal synaptosomes was 25.8% after incubation. These results suggest that both the membranes were fused by the incubation. Hence, we did not carry out further experiments using other types of synaptic membranes.

In an ESR experiment, signals of the probe in the normal synaptosomal membrane were observed after incubation with 12-DSA-labeled liposomes (Table 1). As shown in Table 1, the signal intensities of the 12-DSA in the oxidatively damaged membrane caused by hyperoxia and in the vitamin E-deficient membranes without hyperoxia were significantly decreased. The vitamin E-supplemented rats subjected to hyperoxia did not show a significant decrease in the signal intensity of the 12-DSA in their pre-synaptic plasma membranes after incubation with liposomes containing the 12-DSA. In association with experiments of membrane fusion, the influence of hyperoxia on the fatty acid composition of pre-synaptic plasma membranes after incubation was analyzed. As shown in Table 2, the content of linoleic acid (C\(_{18:2}\)) in synaptosomal membranes from the normal control was increased after incubation by approximately three times. In contrast, docosahexaenoic acid (C\(_{22:6}\)) content was decreased by 11.6%. These alterations in fatty acid contents imply that lipids in both pre-synaptic membranes and liposomal membranes are mixed in membrane fusion. When rats were subjected

![Graph](image_url)

**Table 1.** Changes in ESR signal intensity of 12-DSA in synaptosomal membranes after an incubation with 12-DSA-labeled liposomes.

| Sample           | Signal intensity (cm) | Ratio to Mn\(^{2+}\) signal\(^c\) | Decreasing rate (%) |
|------------------|-----------------------|-----------------------------------|---------------------|
| Normal control\(^a\) | 4.08±0.08             | 0.99                              |                     |
| Hyperoxia\(^b\)  | 3.00±0.02             | 0.82*                             | 17.2                |
| VE-deficient\(^a\) | 3.25±0.11             | 0.85*                             | 14.1                |
| VE-supplement\(^b\) | 3.86±0.06             | 0.97                              | 2.0                 |

All values are means±SE, n=9. \(^a\)Rats were maintained under air. \(^b\)Rats were subjected to hyperoxia.

\(^c\)MnO was used as an external standard. \(^*\)p<0.05 vs. normal control.
to hyperoxia, the arachidonic acid (C20:4) and C22:6 contents in synaptosomal membranes decreased compared to those in normal control membranes. On the other hand, other fatty acid composition, including saturated fatty acids in the membrane, did not change under the experimental conditions (data not shown). When the oxidatively damaged synaptosomes caused by hyperoxia were incubated with the liposomes, changes in the contents of both C20:4 and C22:6 in the synaptosomal membranes were not remarkable. Vitamin E-supplemented rats showed that the content of C18:2 in their pre-synaptic membranes did not change significantly after incubation with liposomes, whether they were subjected to hyperoxia or not (Tables 2 and 3). These results imply that vitamin E prevents the deficit of lipid-mixing in the membrane fusion process caused by lipid peroxidation due to its antioxidant effect.

**DISCUSSION**

The fusion of the neurotransmitter-containing synaptic vesicles with pre-synaptic plasma membranes is recognized to be essential for neurotransmission in nerve terminals. In this event, specific proteins in synapse such as SNARE including synaptobrevin, syntaxin, SNAP-25, etc. act as an important factor for the docking of both synaptic membranes (13). The denaturation and/or loss of SNARE proteins in the brain have been shown in Alzheimer’s disease, which is characterized by progress deficit of cognitive function (24). This phenomenon may explain the possible mechanisms of deficit of membrane-docking due to degenerated proteins. For the membrane fusion after the docking, membrane lipid components may be involved substantially in the membrane fusion through the lipid-mixing. However, the involvement of lipid peroxides in pre-synaptic membranes, which are produced by oxidative stress and during aging, in the deficit of the fusion is still unclear. In this study, in order to determine whether the oxidative damage of nerve terminals lowers fusion of synaptic plasma membrane with synaptic vesicles in neurotransmission, the influence of oxidative damage in synaptic plasma membranes from rat brains subjected to hyperoxia on membrane fusion with bilayer PC liposomes as a model of synaptic vesicles has been investigated. Although it has been questioned whether hyperoxia induces ROS in living tissues, there are several evidences that ROS, such as hydrogen peroxide and superoxide, are generated by hyperoxia in the lungs.

**Table 2.** Changes in fatty acid composition in pre-synaptic plasma membranes before and after incubation with liposomes.

| Fatty acid | C18:2  | C20:4  | C22:6  |
|-----------|--------|--------|--------|
| PC liposomes | 16.72±0.12 | 4.12±0.06 | nd     |
| Pre-synaptic plasma membranes from normal control rats | | |
| Before incubation with liposomes | 0.58±0.04 | 12.0±0.2 | 21.1±0.4 |
| After incubation with liposomes | 1.92±0.17* | 11.1±0.1 | 18.7±0.2* |
| Pre-synaptic plasma membranes from rats subjected to hyperoxia | | |
| Before incubation with liposomes | 0.55±0.03 | 10.9±0.1** | 18.2±0.2** |
| After incubation with liposomes | 0.64±0.12 | 11.1±0.1 | 16.6±7.5 |

All values are % contents, means±SE; n=9. nd, not detectable. *p<0.01 versus before incubation, **p<0.05 versus before incubation of the membranes from normal controls.

**Table 3.** Changes in fatty acid composition in pre-synaptic membranes from vitamin E-supplemented rats before and after incubation with liposomes.

| Fatty acid | C18:2  | C20:4  | C22:6 |
|-----------|--------|--------|--------|
| Pre-synaptic plasma membranes from vitamin E-supplemented rats | | |
| Before incubation with liposomes | 0.46±0.01 | 11.8±0.05 | 20.1±0.30 |
| After incubation with liposomes | 1.02±0.03* | 11.1±0.11 | 19.6±0.16 |
| Pre-synaptic plasma membranes from vitamin E-supplemented rats subjected to hyperoxia | | |
| Before incubation with liposomes | 0.40±0.03 | 11.8±0.15 | 19.2±0.21 |
| After incubation with liposomes | 1.23±0.20* | 11.1±0.21 | 17.9±0.75 |

All values are % contents, means±SE. n=9. *p<0.01 versus before incubation.
heart muscle, brain and erythrocytes of several animals (25–27), and that the rate of ROS generation in the lung is proportional to the concentration of oxygen inhaled (28).

On the basis of these findings, it is reasonable to consider that the hyperoxia used in this study as an oxidative stress induced ROS in rat brains. This idea is supported by the changes in the activity of ROS-scavenging enzymes in the brain caused by hyperoxia (9). Increases in TBARS and conjugated diene of lipids in pre-synaptic plasma membranes (10, 29) in association with a significant increase in lipid hydroperoxides suggest that oxidative alteration of membrane lipids might be induced by ROS which are generated by hyperoxia (Fig. 2A). Furthermore, protein carbonyl levels, which are formed during oxidation of protein, were accumulated in pre-synaptic plasma membranes through hyperoxia (Fig. 2B). Thus, nerve terminals are damaged markedly by oxidative stress, and hence, the \( \zeta \) potential of the synaptic membrane surface is considered to be decreased. As shown in Fig. 3, the negatively charged potential of the membrane surface in normal control rats was decreased by either hyperoxia or vitamin E deficiency. In contrast, vitamin E-supplemented rats showed that the \( \zeta \) potential of the surface did not change even after hyperoxia. These results suggest that a decrease in the \( \zeta \) potential of the pre-synaptic membrane surface may be caused by oxidized membrane components, i.e. lipid peroxides and oxidized protein, produced by hyperoxia, leading to a decline in depolarization of synaptic plasma membranes after stimulation by KCl. Furthermore, the oxidative alterations in membrane components observed in this study may promote the modification of physical properties of the nerve terminal membranes. In fact, the fluidity of synaptic plasma membrane of rats subjected to hyperoxia becomes lower (4). This idea is supported by a previous report which demonstrated in an in vitro study that isolated synaptosomes are peroxidized by ascorbate-ferrous ion, resulting in a strong modification of the membrane fluidity and lipid composition (30). Results from this study imply that fusion of pre-synaptic plasma membranes with neurotransmitter-containing synaptic vesicles does not take place due to a decline in the lipid-mixing of both synaptic vesicles and pre-synaptic membranes caused by hyperoxia. To verify this idea, synaptosomes isolated from rats subjected to hyperoxia were incubated with liposomes prepared from EPC as a model of synaptic vesicles. Since synaptic vesicles interact at the inner face of pre-synaptic plasma membranes in neurotransmission, in this model experiment of the membrane fusion, it is necessary to allow the evagination of the inner leaflet of the synaptic plasma membrane. The “inside-out” form of pre-synaptic membrane was immunochemically confirmed using a syntaxine antibody (Fig. 1), since syntaxin is an essential protein for the membrane docking between pre-synaptic plasma membrane and synaptic vesicle, and it is located at the inner half of the bilayer of synaptic plasma membranes. Although the probes (R_{18} and 12-DSA) in liposomal membranes were translocated to the lipid core of pre-synaptic plasma membranes isolated from normal control rats, the content of the probes in the peroxidized membranes damaged by hyperoxia or the vitamin E deficiency was significantly decreased (Fig. 4 and Table 1). On the other hand, the vitamin E supplementation inhibited a decrease in a translocation of the probes from liposomes (Table 1).

To confirm that data obtained in these experiments did not reveal the results from an adhesion of liposome to the surface of synaptosomes, the calcein-enclosed liposomes were incubated with the synaptosomes. Since calcein is a water-soluble fluorescent probe, the compound is enclosed inside a core of liposome. If calcein is translocated into the inside core of synaptosome by the membrane fusion, the fluorescence of the probe may be observed after the lysis of the synaptosomes by a surfactant. On the basis of this result, synaptic vesicles fused decidedly with pre-synaptic membranes after incubation. The fatty acid composition of the damaged synaptic plasma membranes did not change after incubation with liposomes, although the composition of \( \mathrm{C}_{18:2} \) and \( \mathrm{C}_{22:6} \) in the membranes isolated from normal control rats changed significantly (Table 2). The inhibitory effect of vitamin E on a decrease in composition of \( \mathrm{C}_{18:2} \) in pre-synaptic membranes after incubation implies that pre-synaptic membranes are protected by its antioxidant efficacy, resulting in decreases of LOOH and oxidized protein formation, and hence, the membrane fusion takes place through the lipid-mixing.

In this study, oxidatively damaged synaptic membranes caused by hyperoxia were used for assessment of the membrane fusion with liposomes. When peroxidized liposomes were also incubated with normal synaptic membranes, a deficit of membrane fusion similar to that mentioned above was observed (data not shown). Consequently, it is obvious that peroxidation of synaptic plasma membranes through oxidative stress induces a deficit of membrane fusion due to a decline of the lipid-mixing.

Thus, our results indicate for the first time that oxidative damage of the synaptic plasma membranes caused by oxygen-derived ROS may interrupt the lipid-mixing, which is one of the mechanisms of the membrane fusion, due to the formation of lipid peroxides and the modified protein in the membranes, resulting in a decrease in the fluidity of membranes (4, 30). Although vitamin E inhibited these abnormal phenomena in this study, other antioxidants have not been assessed for their efficacy on oxidative damage to the synaptic membrane fusion. According to this consideration, it is interesting to note that the oxidative damage to nerve terminals may involve a deficit of the fusion of pre-synaptic plasma membranes with synaptic vesicles in nerve terminals. Further studies, however, are needed to elucidate the influence of ROS on the fusion of pre-synaptic plasma membranes with neurotransmitter-containing synaptic vesicles in nerve terminals.

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