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ORIGINAL ARTICLE

Tocotrienols prevent hydrogen peroxide-induced axon and dendrite degeneration in cerebellar granule cells

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Abstract

It is well known that reactive oxygen species (ROS) attack several living tissues and increase the risk of development and progression of serious diseases. In neuronal level, ROS induce cell death in concentration-dependent fashion. However, little is known about the mechanisms of neuronal changes by ROS prior to induction of cell death. Here we found that treatment of cerebellar granule neurons (CGCs) with 0.5 μM hydrogen peroxide induced axonal injury, but not cell death. The number of dendrites remarkably decreased in hydrogen peroxide-treated CGCs, and extensive beading was observed on survival dendrites. In addition, an abnormal band of the original collapsin response mediator protein (CRMP)-2 was detected by Western blotting in hydrogen peroxide-treated CGCs. Treatment with each tocotrienol isoform prevented axonal and dendrite degeneration and induction of the abnormal band of the original band of CRMP-2 in hydrogen peroxide-treated CGCs. These results indicate that treatment with tocotrienols may therefore be neuroprotective in the presence of hydrogen peroxide by preventing changes to the CRMP-2 that occur before neuron death.

Keywords: ROS, axon, dendrite, autophagy, tocotrienol

Introduction

It is well known that aging is an inevitable and degenerative process. In the process of aging, the cognitive functions of learning and memory gradually attenuate. However, the detailed mechanisms of age-related cognitive dysfunction are not well understood. Recently, several lines of evidence have indicated that age-related cognitive dysfunction is related to oxidative injury in brain. Reactive oxygen species (ROS) including superoxide and hydroxyl radicals, attack living tissues resulting in oxidative injury [1]. The hypothesis that ROS is one actor in the progression

of aging, the ‘free-radical theory of aging’, is well known [2]. Using this theory as a start point, we previously investigated and reported the relationship between ROS-derived oxidative injury and cognitive dysfunction during aging using rodent models. We observed gradual, age-dependent degeneration in cognitive functions in several maze tasks [3]. In addition, the cognitive performance scores of vitamin E-deficient young rats and ROS-exposed young rats were similar to those of normal (not vitamin E-deficient or ROS-exposed) older rats. We also found apoptosis and β -amyloid-like proteins in the

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CA1 region of the hippocampus in these three models [4]. These results strongly indicate that age-related cognitive dysfunction is associated with ROS. However, it is difficult to identify possible treatments for or means of preventing cognitive dysfunction, as the dysfunction manifests only after the neurons have already died. It is necessary to find early events of neuronal change before the induction of cell death in ROS-exposed neurons.

In order to detect early changes in ROS-exposed neurons, we focused on axon and dendrite function, which plays an important role in neurotransmission. A large number of synapses exist on the distal ends of axons, and many substances, including neurotransmitters and other proteins, are carried from the cell body by the axonal transport system. Axonal dysfunction may therefore have serious effects on cognitive function even if cell death does not occur. Using electron microscopy, Urano et al. examined a large number of synaptic vesicles accumulated in synapses in brain slices from aged rats and ROS-exposed young rats [5]. Touma et al. reported that neurite, which is characterized by expansion and contraction, degenerated in nerve-growth factors (NGF)-free medium [6]. These findings indicate that axons are more sensitive than the cell body, because of its flexibility [6,7]. On the other hands, dendrites are receivers of neurotransmitters. Many kinds of receptors exist on the post-synaptic region. Thus, we focused first on axon and dendrite function.

If ROS induce axonal dysfunction, treatment with antioxidant substances may affect neurons. To clarify the possibility of preventing ROS-induced axonal dysfunction, we used tocotrienols. Tocotrienols, which include four different isoforms (α -, β -, γ -, and δ -tocotrienol), comprise a vitamin E family (Figure 1). Vitamin E is one of the most common natural lipophilic vitamins. One of pivotal functions of vitamin E is as an antioxidant. Vitamin E plays a role in radical scavenger [8–10]. It has been reported that palm and olive oils contain relatively large amount of vitamin E, and widely mixed in food products as

an antioxidant substances [9,11]. Recently, several lines of evidence have suggested that vitamin E has neuroprotective effect [12,13]. Furthermore, the antioxidant or non-antioxidant function of tocotrienols has proven more effective than that of tocopherols [14,15]. However, the neuroprotective effects of tocotrienols at the cell biology level are not well understood.

The purpose of this study was to determine whether treatment of primary cultures of mice cerebellar granule cells (CGCs) with a low concentration of hydrogen peroxide induces axonal degeneration and to determine the neuroprotective effects, if any, of α - and γ -tocotrienol in low concentration hydrogen peroxide-treated CGCs. We measured collapsin response mediator protein (CRMP)-2, which is part of a family of cytoplasmic proteins in the brain, with important roles in neurite polarity and axon guidance [16–18]. Furthermore, we investigated the autophagy-function of axonal regions in low concentration hydrogen peroxide-treated CGCs. Our data illustrate that ROS-derived axon and dendrite dysfunction is one of the early signs of ROS-induced cell degeneration. ROS-derived axon and dendrite degeneration, but not cell death, may be one of the processes underlying age-related cognitive dysfunction. Resulting from these investigations may help to prevent neurodegenerative diseases related to axonal dysfunction.

Materials and methods

Animals and reagents

The wild-type C57BL/6 mouse strains were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The α - and γ -tocotrienols were kindly provided by Eisai Food & Chemical Co., Ltd (Tokyo, Japan). All other chemical agents were obtained from either Wako Pure Chemicals Industries, Ltd. (Osaka, Japan) or Sigma-Aldrich Corporation (St. Louis, MO, USA). All animal experiments were performed with the approval of the Animal Protection and Ethics Committee of the Shibaura Institute of Technology. All tissue culture plates and dishes were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). All other reagents were purchased from Sigma-Aldrich.

Cell cultures

Primary cultures of CGCs were prepared from 7-day-old postnatal mice as described previously, with some modifications [19]. Briefly, the cerebellum was dissected after decapitation, cleaned free of meninges, and then treated with 0.125% (w/v) trypsin in minimum essential medium (MEM) for 10 min at 37°C. The reaction was terminated by adding feed medium consisting of MEM, 25 μ g/mL streptomycin, 25 U/mL penicillin, and 5% heat-inactivated fetal calf serum

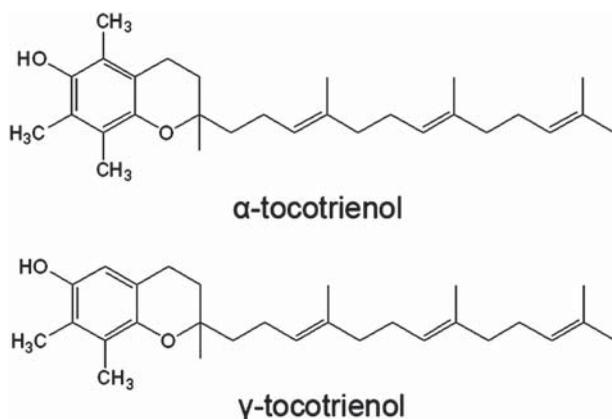


Figure 1. Chemical structure of tocotrienols.

(FCS)(Biological Industries, BeitHaemek, Israel) supplemented with K^+ to a final concentration of 25 mM. After trituration, the cells were suspended and plated onto 6- or 24-well dishes coated with 0.1% polyethyleneimine. This was designated as Day 0 (DIV0). On Day 1, cytosine-D-arabino-furanoside was added to a final concentration of 1 μ M to prevent non-neuronal cell proliferation. CGCs were used in experiments on Day 3.

Analysis of lipid peroxidation

For analysis of lipid oxidation, we measured lipid hydroperoxides (LOOH) and thiobarbituric acid reactive substances (TBARS) after treatment of CGCs with hydrogen peroxide in the presence or absence of each tocotrienol isoform. LOOH were measured using chemiluminescence methods, as described previously with some modifications [20]. One hundred microliters of cell lysate was extracted with a mixture of chloroform and methanol (2:1 v/v, 1 mL), and evaporated by N_2 gas. The residue was dissolved by 200 μ L of methanol, and mixed with 150 μ L of a chemiluminescent solution (a mixture of 0.18 mg isoluminol/mL and 1 mg micropoxidase/mL of 70% methanol, 150:1, v/v). Chemiluminescence intensity was analyzed using a Luminometer (Luminescencer-PSN AB-2200, Atto Corp., Tokyo, Japan). Preparation of the standard curve for the analysis was carried out using cumenhydroperoxide as a standard for LOOH. Lipid peroxides were measured by Yagi's method, as previously described with some modifications [21]. One hundred microliters of cell lysate was mixed with 200 μ L of 5 mM EDTA solution, 2 mL of 1% phosphate acid solution, and 1 mL of 0.7% thiobarbituric acid solution. The mixture was heated in a block heater for 45 min at 100°C. After cooling in an ice box, the sample was then incubated with 2 mL of butanol for 3 min. After centrifugation, we isolated the upper layer and measured the absorbance of 535 nm using spectrophotometry (GeneQuant 100, GE Healthcare UK Ltd, Buckinghamshire, UK). Both oxidized markers were evaluated per mg protein in the samples and were performed at least five times. Data were analyzed using Student's *t*-test, with findings of $p < 0.01$ considered significant.

Transfection

CGCs were transfected using Lipofectamin 2000 (Life Technologies Japan Ltd, Tokyo Japan) using the manufacturer's protocol. One day before transfection, medium was changed without antibiotics. A pEGFP-C1 mammalian expression vector (Clontech Takara Bio Inc., Tokyo, Japan) was added to 25 μ L of opti-MEM I Life Technologies reduced serum medium. Lipofectamine 2000 was diluted with 25 μ L of opti-MEM I reduced serum medium. After 5 min incubation, the diluted

DNA was combined with diluted Lipofectamin 2000. After 15 min, the solution was added drop-wise to the dishes. After 3 h, the solution was added to the culture medium, and used in the experiment. The transfected cells were observed with a fluorescence microscope (IX81, Olympus, Tokyo, Japan). Transfection experiments were performed at least three times.

Axon and dendrite degeneration

Axon or dendrite degeneration was evaluated by monitoring morphological hallmarks of neurite degeneration such as beading and fragmentation, as described previously with some modifications [19]. To evaluate the extent of axons, pEGFP-C1 vector was transfected to CGCs (see Materials and methods). After 18 h of transfection, CGCs were treated with 0.5 μ M hydrogen peroxide in the presence or absence of 5 μ M α - or γ -tocotrienol. Twenty-four hours after treatment with hydrogen peroxide, the cells were fixed with 4% paraformaldehyde (PFA) (Merck KGaA, Darmstadt, Germany) in PBS for 15 min at 4°C. Photomicrographs of the cells were taken on an Olympus IX81 phase-contrast microscope (Olympus) equipped with a DP72 digital camera (Olympus), stored and then processed on a personal computer. Axon and dendrite degeneration experiments were performed at least three times.

Western blotting

After 24 h of treatment with hydrogen peroxide, the samples were collected and used in Western blotting as described previously, with some modifications [6]. The lysates were centrifuged, and protein content was determined using a Bio-Rad protein assay (#500-0006JA, Bio-Rad Japan, Tokyo, Japan) according to the manufacturer's procedure. Protein extracts (15 μ g) were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon transfer membranes (PVDF; Merck KGaA). The membranes were washed and then incubated in blocking solution [Tris-HCl-buffered saline, pH 7.6 (TBS), containing 0.1% Tween20 and 3% bovine serum albumin (BSA)] for 1 h at room temperature (R/T). The membranes were washed in TBS containing 0.1% Tween20, and then treated with anti-human CRMP-2 (C4G) mouse IgG monoclonal antibody (#11096, Immuno-Biological Laboratories Co., Ltd, Gunma, Japan) at 1:400 dilution overnight at 4°C. Anti-mouse IgG HRP antibody (Promega Corp., Madison, WI, USA) was used as a secondary antibody at 1:4000 dilution for 1 h at R/T.

In LC3 assay, the protein extracts (15 μ g) were separated on 15% SDS-polyacrylamide gels. The transferred membranes were treated with anti-human LC3 (APG8B)(N-term) rabbit IgG polyclonal antibody (#AP1802a, Abgent, Inc., CA, USA)

at 1:400 dilution overnight at 4°C. Anti-rabbit IgG HRP antibody (Promega) was used as a secondary antibody at 1:2000 dilution for 1 h at R/T. Both Western blotting experiments were performed at least three times. All chemiluminescent signals were generated by incubation with the detection reagents (ECL Prime Western Blotting Analysis Reagent; GE Healthcare) according to the manufacturer's procedure. For normalization of each band of CRMP-2 and LC3, the membranes were reprobbed with anti- β actin antibodies (#ab8226, Abcam Plc., Cambridge, UK). The relative intensities of CRMP-2 and LC3 were determined using LAS-3000 (FUJIFILM Corp., Tokyo, Japan).

Immunocytochemic analysis

After 24 h of treatment with hydrogen peroxide, we detected autophagy using a Autophagy/Cytotoxicity Dual Staining Kit using the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). For judgment of cell death, the samples were treated with propidium iodide (PI) solution at 1:1000 dilution for 2 min at R/T. After PI staining, the samples were treated with monodansylcadaverine (MDC) solution at 1:1000 dilution for 10 min at 37°C. Tamoxifen, a known inducer of autophagy vacuoles, was used as a positive control. The stained cells were observed with a fluorescence microscope. Immunocytochemical experiments were performed at least three times.

Results

Treatment with hydrogen peroxide induces lipid peroxidation in CGCs

To determine the effect of a low concentration of hydrogen peroxide, we checked the lipid peroxidation

levels in CGCs. LOOH and TBARS were used as indices of lipid peroxidation. After treatment with 0.5 μ M hydrogen peroxide, the levels of LOOH significantly increased (Figure 2A). Treatment with each tocotrienol isoform significantly inhibited the levels of LOOH in hydrogen peroxide-treated CGCs. After treatment with hydrogen peroxide, TBARS did not significantly differ in the presence or absence of each tocotrienol isoform (Figure 2B). These results indicate that treatment with a low concentration of hydrogen peroxide induced lipid hydroperoxides in CGCs.

Appearance of axon and dendrite injury after treatment of pEGFP-transfected CGCs with hydrogen peroxide

Treatment with 0.5 μ M hydrogen peroxide of CGCs induced abnormal morphology and marked axonal beading (Figure 3A). Fragmented nuclei were not found on Hoechst 33258 staining (data not shown). However, treatment with 5 μ M α - or γ -tocotrienol significantly prevented axonal beading on hydrogen peroxide-treated CGCs. This finding indicates that treatment with 0.5 μ M hydrogen peroxide induced axonal degeneration of CGCs, but did not induce cell death. After treatment with 0.5 μ M hydrogen peroxide, the distal end of the axons appeared to be collapsed. Furthermore, we investigated morphological changes in the dendrites around the cell body. Large numbers of dendrite were observed in a spider-web formation around the cell body (Figure 3B). Whereas these dendrites were densely distributed around the cell bodies of normal CGCs, the number of dendrites was lower in hydrogen peroxide-treated CGCs. In surviving dendrites, extensive beading was observed. Treatment with each tocotrienol isoform prevented dendrite degeneration in the hydrogen peroxide-treated CGCs. These results indicate that treatment

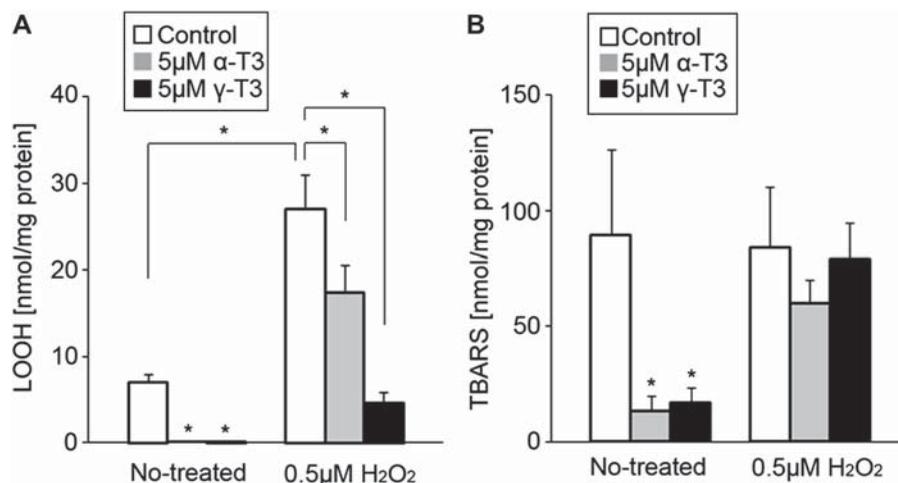


Figure 2. Analysis of lipid peroxidation. CGCs were treated with 0.5 μ M hydrogen peroxide in the presence or absence of α -tocotrienol (5 μ M) or γ -tocotrienol (5 μ M). After 24 h, the cells were collected and measured to determine the index of lipid peroxidation (LOOH is shown in (A), TBARS is shown in (B)). Both oxidized markers were evaluated per mg protein in samples. Each column represents the mean of results of five independent experiments. Data were analyzed by Student's t-test, with findings of $p < 0.01$ considered significant.

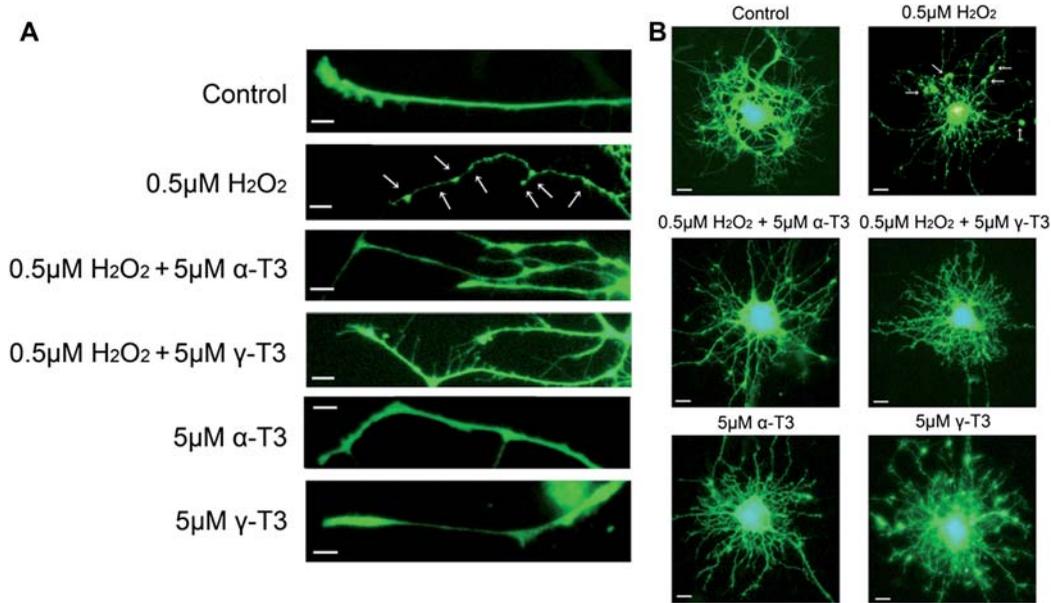


Figure 3. Axon and dendrite degeneration were induced by treatment of EGFP-transfected CGCs with hydrogen peroxide. Each image was taken by fluorescence microscopy. After 18 h of transfection with EGFP, the CGCs were treated with 0.5 μM hydrogen peroxide in the presence or absence of α -tocotrienol (5 μM) or γ -tocotrienol (5 μM). After 24 h, the cells were fixed with 4% PFA in PBS. Photomicrographs of the cells were taken and analyzed in the regions of the axon (A) and the dendrite (B) on a personal computer. The scale bar is 10 μm . Arrows indicate beading on the degeneration of CGCs. At least, three wells were used per experiment. The method of transfection is described in the Materials and Methods.

of CGCs with a low concentration of hydrogen peroxide induces axonal and dendritic degenerations, but not cell death.

Hydrogen peroxide induced CRMP-2 alteration on western blot analysis

On Western blotting, two original bands of CRMP-2 (64- and 72-kDa) were detected for a mouse brain homogenate sample on the data sheet of IBL Co., Ltd (<http://www.ibl-japan.co.jp/eng/index.htm>). We detected the two original bands of CRMP-2 in CGCs. However, the upper side of the original two bands of CRMP-2 was very weak in our experimental model. Treatment of CGCs with hydrogen peroxide enhanced an unusual band located below the two original bands of CRMP-2 in a concentration-dependent fashion (Figure 4A). Treatment with α - or γ -tocotrienol significantly inhibited the appearance of this unknown band of CRMP-2. These results indicate that tocotrienols protect axon and dendrite functions while preventing the induction of the unknown CRMP-2 in hydrogen peroxide-treated CGCs.

To clarify the relationship between the induction of the unknown band of CRMP-2 and axonal beading after treatment in hydrogen peroxide-treated CGCs, we focused on a cytoskeletal protein. The cells were treated with colchicine, which is used as an inhibitor of microtubule polymerization. Previously, we found that treatment of neuro2a cells with colchicine induced extensive axonal beading [22].

Treatment with colchicine induced an unusual band located below the two original bands of CRMP-2 in a concentration-dependent fashion (Figure 4B). These results indicate that treatment with hydrogen peroxide induced axonal degeneration via induction of an unknown band of the CRMP-2. Induction of

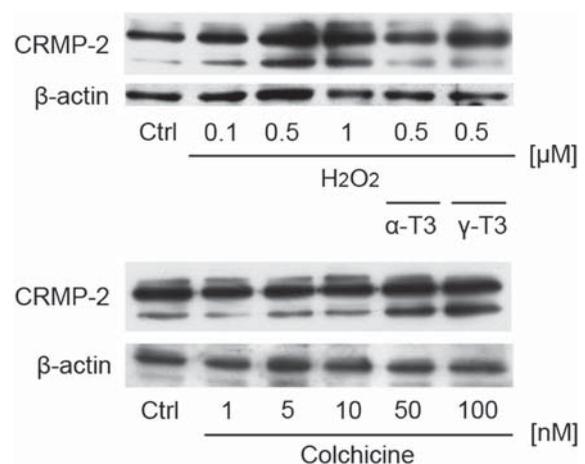


Figure 4. Denatured CRMP-2 proteins appeared after treatment of CGCs with hydrogen peroxide. CGCs after treatment with hydrogen peroxide in the presence or absence of α -tocotrienol (5 μM) or γ -tocotrienol (5 μM) were lysed and used for Western blotting analysis. The same membranes were re probed and used for the detection of β -actin (A). CGCs were treated with various concentrations of colchicine (B). After 24 h, the cells were lysed and used for Western blotting. Each data represents the mean of three independent experiments.

axonal beading may affect dysfunction of microtubule assembly.

Changes in MDC accumulation and LC3 expression in hydrogen peroxide-treated CGCs

To more precisely determine the mechanism by which hydrogen peroxide induces axonal beading in CGCs, we examined autophagy using immunocytochemical and Western blot analysis. MDC, a fluorescent compound, was used as a probe for detection of autophagic vacuoles in cultured cells [23]. In Figure 5A, CGCs

tested with 0.5 μM of hydrogen peroxide were stained with MDC. Treatment of CGCs with 1 μM hydrogen peroxide did not result in positive MDC staining. However, staining with PI, which is a hall-mark of cell death, was positive. Treatment with each tocotrienol isoform prevented accumulation of MDC in 0.5 μM hydrogen peroxide-treated CGCs.

To more determine about autophagy in hydrogen peroxide-treated CGCs, we checked expression of LC3 protein level on Western blotting (Figure 5B). Treatment of CGCs with 1 or 5 μM Tamoxifen increased LC3 expression. Treatment of CGCs with

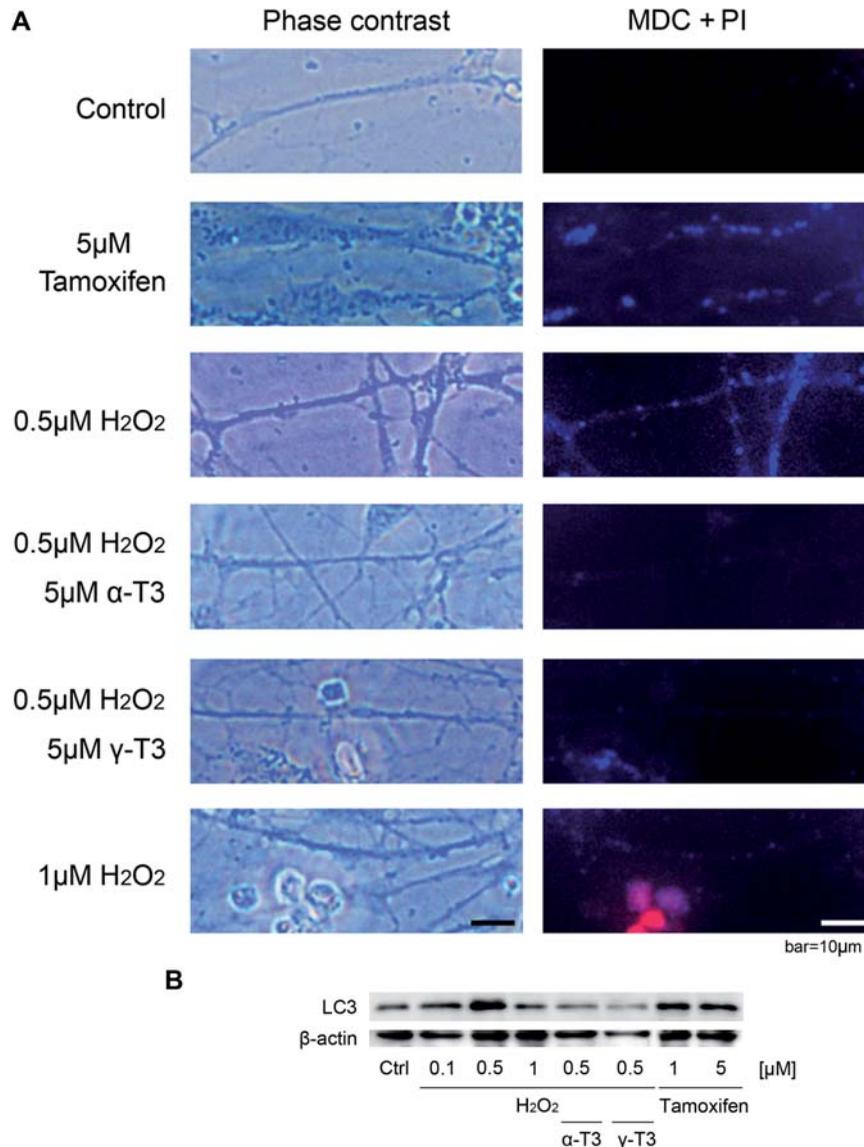


Figure 5. Treatment with hydrogen peroxide induced autophagy in CGCs, and its prevention by tocotrienols. Immunocytochemical analysis of CGCs using the double staining system of MDC and PI. CGCs were treated with 0.5 μM of hydrogen peroxide in the presence or absence of α -tocotrienol (5 μM) or γ -tocotrienol (5 μM). After 24 h, the cells were treated with PI solution (1:1000) for 2 min at R/T. After PI staining, the cells were treated with MDC solution (1:1000) for 10 min at 37°C. Treatment with 5 μM Tamoxifen was used as a control. Photomicrographs of the cells were taken and analyzed on a personal computer. At least, three wells were used per experiment. The scale bar is 10 μm (A). CGCs after treatment with hydrogen peroxide in the presence or absence of α -tocotrienol (5 μM) or γ -tocotrienol (5 μM) were lysed and used for Western blotting analysis. The same membranes were re-probed and used for the detection of β -actin. Two different concentrations of Tamoxifen were used as a control of autophagy. After 24 h, the cells were lysed and used for Western blotting (B). Each data represents the mean of three independent experiments.

0.5 μM hydrogen peroxide, the expression of LC3 was stronger than that of 0.1 μM one. On the other hands, treatment with each tocotrienol isoform prevented enhancement of LC3 expression in 0.5 μM hydrogen peroxide-treated CGCs. Both results indicate that treatment with a low concentration of hydrogen peroxide leads to the accumulation and induction of autophagic vacuoles, but not cell death.

Discussion

Treatment with a low concentration of hydrogen peroxide gradually but certainly induces lipid peroxidation in CGCs

It is well known that hydrogen peroxide attacks living tissues. In the present study, we used hydrogen peroxide not only to examine its direct effects on the cell membrane, but also as a trigger for ROS production in the cell. The purpose of this study was to determine the early events of ROS-induced cell degeneration before the induction of cell death. Treatment with 0.5 μM hydrogen peroxide did not induce cell death. However, the levels of LOOH, but not TBARS, significantly increased in hydrogen peroxide-treated CGCs (Figure 2). ROS, including superoxide, hydroxyl radical, hydroperoxyl radical and hydrogen peroxide, attack polyunsaturated fatty acid, and play a crucial role in initiating lipid peroxidation. As a result of this reaction, i.e., a chain reaction of lipid peroxidation, LOOH increases [1,24]. Finally, products of aldehyde substances detected by TBA methods are increased. LOOH may increase more quickly than TBARS. We also analyzed antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The activities of these enzymes did not change in hydrogen peroxide-treated CGCs (data not shown). These results suggest that treatment with 0.5 μM hydrogen peroxide slowly but certainly induce cell oxidation in CGCs without affecting the functions of antioxidant enzymes.

Hydrogen peroxide induces axon and dendrite degeneration before induction of cell death in pEGFP-transfected CGCs

The density of CGCs in culture is typically very high, and the extended axons of CGCs are very long, more than 20 times longer than the diameter of the cell body. To evaluate cell morphology, the pEGFP-C1 mammalian expression vector was transfected to CGCs using the lipofection method. The transfection efficiency was low, at 10–20%. However, this low transfection efficiency made it convenient for us to check the condition of whole cells. We observed the cell morphology of individual cells from the distal end of the axon to the cell body.

After 18 h of transfection, treatment of CGCs with hydrogen peroxide resulted in extensive axonal beading (Figure 3A). Axonal beading is a major hall mark of neurite degeneration [6,19]. In addition, the number of dendrites was remarkably decreased, with particularly extensive beading around surviving dendrites in hydrogen peroxide-treated CGCs (Figure 3B). It is well known that dendrites are receivers of neurotransmitters. Impairment of axonal and dendritic functions such as damaged neurotransmitter receptors, dysfunction of axonal transport systems and other discrepancies may occur in hydrogen peroxide-treated CGCs. In our present study, both the pre- and post-synaptic sides of synapses were injured in CGCs after treatment with hydrogen peroxide.

Treatment with each tocotrienol isoform prevented axon and dendrite degeneration in hydrogen peroxide-treated CGCs due to their antioxidant effects. Before start of this study, we checked cell survival on hydrogen peroxide-treated neuro2a cells in the presence or absence of each vitamin E isoforms. The preventive effects of tocotrienol were significantly stronger than that of tocopherol. Usually, vitamin E exist in cell membrane because of its hydrophobic formation. Serbinova et al. [25] reported that antioxidant activity of α -tocotrienol is 40–60 times higher than that of α -tocopherol against (Fe^{2+} + ascorbate)- and (Fe^{2+} + NADPH)-induced lipid peroxidation in rat liver microsomal membranes. They explained that tocotrienol is more uniformly distributed in membrane bilayer because of its unsaturated isoprenoid chain. In this study, treatment with each tocotrienol isoform significantly prevented induction of LOOH in hydrogen peroxide-treated CGCs (Figure 2A). These results strongly suggest that ROS is one of the reasons of the induction of axon and dendrite beading in our experimental model. However, further investigation is needed to determine the relationship between hydrogen peroxide-induced axon and dendrite degeneration and the possibility of impairment of the neurotransmission system. We did not observe fragmented and accumulated nuclei using Hoechst33258 staining. Chronic exposure to ROS, even at low concentrations, may gradually increase the risk of neuronal degeneration.

Treatment with hydrogen peroxide induces unusual CRMP-2 in CGCs

In order to clarify the mechanism of induction of axon and dendrite degeneration in hydrogen peroxide-treated CGCs, we analyzed CRMP-2 using Western blotting. CRMP-2, a cytosolic protein, is highly expressed in the distal ends of axons and plays a crucial role in neurite polarity and axon guidance [26–28]. Inagaki et al. reported that overexpression of the CRMP-2 induces multiple axons

in cultured hippocampal neurons [29]. Treatment with hydrogen peroxide induced an unknown band of the CRMP-2 in a concentration-dependent fashion (Figure 4A). Treatment with each tocotrienol isoform prevented induction of the unknown band of the CRMP-2. This result showed that treatment with ROS can directly or indirectly induce changes in the CRMP-2. Jiang et al. examined CRMP-2 where cleavage by ischemia-activated calpain and synaptosomal CRMP-2 were more sensitive to cleavage compared with the cytosolic fraction [27]. Touma et al. reported that the CRMP-2, which is cleaved by calpain 1 in the presence of calcium ions, induces neurite degeneration in mice [6]. Our results may be one of the early events caused by ROS before the induction of cell death in our experimental model. Recently, several lines of evidence highlight the relationship between changes in the CRMP-2 and the progressive neurodegenerative effects characteristic of Alzheimer's disease (AD) [30–32], ischemia [27] and other disorders [28,33]. Furthermore, Castegna et al. reported that the CRMP-2 is significantly oxidized in the AD brain, and that ROS attenuate the function of the CRMP-2 [34]. Consistent with this evidence, our hypothesis suggests that treatment with hydrogen peroxide induces dysfunction in the CRMP-2. However, further investigation is needed to elucidate the relationship between dysfunction of axons and dendrites and ROS-derived CRMP-2 alteration in neurons.

We focused on microtubules in order to obtain more evidence about the induction of the unknown CRMP-2 in hydrogen peroxide-treated CGCs. Microtubules play important roles in axonal extension [26,29,35]. Recently, several lines of evidence have been reported about the importance of the relationship between the CRMP-2 and microtubules [18,36,37]. Uchida et al. reported CRMP-2 which cleaved by calpain bound to α -tubulin [38]. If changes in the CRMP-2 are one of the reasons for axonal beading on hydrogen peroxide-treated CGCs, treatment with hydrogen peroxide also induces dysfunction of cytoskeletal protein. We used colchicine, which acts as an inhibitor of microtubule polymerization by binding to tubulin. Previously, we found extensive neuronal beading in colchicine-treated neurons [22]. On Western blotting, treatment with colchicine gradually increased induction of the unknown band of the CRMP-2 in a concentration-dependent fashion (Figure 4B). This result was similar to that of hydrogen peroxide-treated CGCs. These results indicate that treatment with low concentrations of hydrogen peroxide induces some changes in microtubule polymerization via induction of the CRMP-2 alteration. We are continuing to investigate the relationship between possible disruption of microtubule assembly and ROS-derived axon and dendrite degeneration.

Treatment with hydrogen peroxide affected autophagy in CGCs

To determine whether transport systems were damaged in the axons of hydrogen peroxide-treated CGCs, we assessed autophagy using immunocytochemical and Western blot analysis. In Figure 5, treatment with 0.5 μ M hydrogen peroxide remarkably increased MDC and LC3 in CGCs. Accumulation of autophagic vacuoles is related to axonal beading, and the regions of axonal beading corresponded to the location of autophagic vacuoles in the NGF deprivation model of PC12 cells [39]. In our experimental model, treatment with 0.5 μ M of hydrogen peroxide induced extensive axonal beading.

Several lines of evidence have shown that autophagy is associated with aging [40–43]. Saimonsen et al. discussed the relationship between changes in autophagy levels and accumulation of age-related oxidative damage [41]. Down-regulation of transcriptional levels of autophagy may contribute to normal human brain aging [42]. Following the free radical theory of aging, low concentrations of hydrogen peroxide and involvement of dysfunction of autophagy may induce the accumulation of autophagy vacuoles via specific damage to axon. Treatment with hydrogen peroxide at concentrations higher than 1 μ M results in immediate cell death for some cells, and surviving cells may no longer have normal axonal function, including autophagy, as the axons accumulate oxidative damage by ROS. However, further investigation is needed to determine changes in the levels of autophagic vacuoles and ROS-derived axonal damage. Little is known about the relationship between vitamin E and autophagy. We are continuing to study the relationship between the neuroprotective effect of tocotrienols and changes in ROS-derived autophagy in neuronal cells.

Conclusion

In summary, treatment with a low concentration of hydrogen peroxide induces axon and dendrite degeneration, but not cell death. It is possible that one of the reasons for the induction of axonal degeneration is changes in the CRMP-2 and disruption of microtubules on hydrogen peroxide-treated CGCs. Treatment with each tocotrienol isoform inhibited axon and dendrite degeneration. These results showed that chronic exposure to ROS, even at low concentrations, may gradually accelerate neuronal degeneration. Several lines of evidence have shown that the CRMP-2 is phosphorylated in Alzheimer's disease [30–32]. In the near future, we plan to examine the relationship between ROS-derived axon and dendrite degeneration and neurodegenerative disorders such as cognitive impairment and Alzheimer's disease.

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Declaration of interest

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