Alpha-tocopherol protects H_2O_2-induced tight junction occludin disruption

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ABSTRACT

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Vitamin E is the most important lipid-soluble antioxidant in humans. Although alpha-tocopherol is suggested that it has protective effect from many diseases, little is known about the prevention of occludin alteration in tight junction of blood-brain barrier (BBB) under pathologic insults producing reactive oxygen species (ROSs). In this study, the effects of alpha-tocopherol on H_2O_2-induced tight junction occludin were studied with primary culture of rat brain microvessel endothelial cells. Alpha-tocopherol had no apparent cytotoxicity up to 2.8 mM. The preincubation with alpha-tocopherol suppressed the H_2O_2-induced cytotoxicity in AlamarBlue assay and phase contrast microscopy. In confocal laser microscopy and western blot, H_2O_2-induced loss of occludin was suppressed by preincubation with alpha-tocopherol. The present findings provide evidence that alpha-tocopherol may be beneficial for cellular

protection from pathologic insults. Since alpha-tocopherol was demonstrated to have far fewer adverse effects, it would become a noteworthy nutrient or drug for treatment of neurodegenerative diseases.

Key Words : alpha-tocopherol, BBB, tight junction, occludin, H_2O_2

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I. Introduction

The blood-brain barrier (BBB) is a complex biological system that consists of endothelial cells, pericytes and astrocytes, which are involved in the induction and maintenance of its physiological and ultrastructural characteristics. The BBB plays a primordial role in isolating the cerebral parenchyma as well as in controlling brain homeostasis by its selective permeability to nutrients and other molecules flowing through the cerebral microcapillaries[1]. The endothelial cells forming BBB are highly specialized to allow precise control over the substances that leave or enter the brain. Electron microscopic studies revealed several major factors that distinguished brain endothelial cells from their peripheral relatives[2]. First, they contain lower amounts of endocytotic vesicles, and second, the space between adjacent cells is sealed by tight junctions; both factors restrict intercellular flux[3].

An elaborate network of complex tight junctions (TJs) between the endothelial cells forms the structural basis of the BBB and restricts the paracellular diffusion of hydrophilic molecules[4]. Once thought to be static structures, TJs are in fact regulated in both physiological and pathological states[5], and changes in TJ protein expression and/or organization have been associated with altered permeability[6,7,8].

TJs are complexes of plasma membrane proteins connected to the cytoarchitecture via membrane associated accessory proteins. Claudin, copolymerized occludin, and junctional adhesion molecule are integral membrane proteins interacting with those of neighboring plasma membrane and form TJ barrier[9,10]. Cytoplasmic TJ accessory proteins [Zonular occludens (ZO)-1, ZO-2, cingulin] connect TJs to the actin cytoskeleton[11].

Signal transduction pathways that are triggered by bacterial toxins and cytokines modulate the function of TJs[12,13]. Response to external stress and signaling is regulated by 3 major mitogen-regulated protein kinase system : extracellular signal regulated kinase and 2 stress-activated protein kinases p38 and c-Jun N-terminal kinase[14,15,16].

Reactive oxygen species (ROSs) are implicated in the pathophysiology of variety of vascular diseases, ischemia-reperfusion and inflammation. ROSs including superoxide (O_2^-) and hydrogen peroxide (H_2O_2) can alter the function of specific proteins and enzymes[6,17]. The blood level of ROSs also assumed to play an important role in disease process[18,19]. In most cases, the mechanism by which these agents

interact with their molecular targets in BBB is still unknown.

Alpha-tocopherol is the most important lipid-soluble antioxidant in humans. Although alpha-tocopherol is suggested that it has protective effect from many diseases[20,21], little is known about prevention of occludin alteration under pathologic insults producing ROSs. In this study, the protective effects of alpha-tocopherol on H_2O_2-induced TJ occludin were studied with primary culture of rat brain microvessel endothelial cells.

II. Materials and Methods

1. Materials

Cell culture plates were purchased from Corning Costar (Acton, MA, USA). Collagenase/dispase was purchased from Roche Applied Science (Indianapolis, IN, USA). Minimal essential medium (MEM), DMEM/F12, and rat-tail collagen (type I) were purchased from Invitrogen (Carlsbad, CA, USA). Alpha-tocopherol, all other nutrients, salts, antibiotics, etc. used in culture media were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-occludin, goat anti-rabbit IgG-HRP, and goat anti-rabbit IgG-FITC were purchased from Zymed Laboratories (San Francisco, CA, USA).

2. Isolation of rat brain microvessel endothelial cells

Rat brain microvessel endothelial cells (RBMECs) were isolated from cerebrums of 4-week old Sprague Dally rats (Samtaco, Seoul, Korea) by enzymatic digestion as described in other report[22] with minor modification. In short, twenty brains were placed in ice-cold MEM (pH 7.4) buffered with 50 mM HEPES, containing 100 μg/ml penicillin/streptomycin. Brainstem, meninges and large surface vessels were removed from brains. Cerebrums were collected and minced with scalpel blades into approximately 1-mm segments. The minced sample was digested with

collagenase/dispase (0.5 mg/ml) for 1 hr at 37°C with shaking. Resulting homogenate was centrifuged at 2000 × *g* for 10 min at room temperature (RT). Following removal of the supernatant, the remainder was resuspended in 13% (w/v) dextran solution and centrifuged at 9000 × *g* for 10 min. The pellets were further incubated with collagenase/diapase (1 mg/ml) for 2 hr at 37°C with shaking. Cells were sedimented at 1000 × *g* for 10 min. The pellets were resuspended with DMEM (pH 7.4), loaded on a 50% (v/v) Percoll gradient centrifugation (39,200 × *g*, 1 hr), and centrifuged at 1700 × *g* for 10 min. RBMECs in the second band were collected and washed two times to remove Percoll with DMEM (pH 7.4) by centrifugation (1000 × *g*, 10 min). Finally, RBMECs were suspended with plating medium (DMEM/F12 containing 20% horse serum, 100 μg/ml penicillin/streptomycin, 45 μg/ml polymyxin B, and 1.25 mg/ml amphotericin B) with DMSO (10%) and stored overnight at −70°C.

3. Culture of rat brain microvessel endothelial cells

Cell viability was determined by trypan blue exclusion test. RBMECs were seeded at a density of 1×10^5 cells/cm^2 in 96-well, 24-well, 6-well plates, and chamber slides. The culture surfaces were treated with rat-tail collagen (50 μg/ml). RBMECs were cultured in plating media. The cells were grown in a 37°C incubator in 5% CO_2 and 95% room air. After the first 3 days, the culture medium was changed every other day with changing media (DMEM/F12 containing 20% horse serum, 100 μg/ml penicillin/streptomycin, and 100 μg/ml heparin). The growing cells were

observed with phase-contrast microscope (CK2, Olympus, Tokyo, Japan).

4. Experimental design

Sub-confluent cells (about 80 %) were used on each experiment. Five sets of experiments were performed at standard culture conditions. Alpha-tocopherol was dissolved in ethanol and incubated in serum for 1 hr. The final concentration of ethanol in culture medium was 0.01%.

5. Cell viability

Viability of RBMECs was assessed using AlamarBlue assay (Serotec, Kidlington, Oxford, UK). The absorbance was measured at wavelength of 570 nm using Spectra MAX 340 (Molecular Devices, Sunnyvale, CA, USA). Background absorbance measured at 600 nm was subtracted from the 570 nm absorbance (n = 5). Viability was expressed as a percentage of control.

6. Confocal laser microscopy

RBMECs grown on chamber slide (Nunc, Roskilde, Denmark) were exposed to alpha-tocopherol and H_2O_2 as previously described. After the culture medium was removed, monolayer was washed with prewarmed phosphate-buffered saline (PBS, 0.01 M). Cells were fixed with 3% paraformaldehyde (in PBS) for 20 min at RT and

permeabilized with 0.1% Triton X-100 (in PBS) for 10 min at RT. After fixing and permeabilization, monolayer was blocked with 1% bovine serum albumin (BSA)/PBS for 60 min at RT. Confluent monolayers from each treatment group were incubated with anti-occludin (5 μg/ml) primary antibody for 1 hr at RT. The cells were rinsed with 1% BSA/PBS, followed by incubation with FITC conjugated with secondary antibodies (5 μg/ml) for 1 hr at RT in the dark. The fluorescent-stained cells were rinsed three times with PBS before being mounted with cover glass with 50% glycerol-PBS and sealed. Photographs were taken with a confocal laser microscope (LSM-510 meta, Zeiss, Berlin, Germany).

7. Western blot

Cell lysates were prepared from RBMEC monolayers by adding Triton/deoxycholate/SDS buffer (0.2% SDS, 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 2 mM EDTA, 10 mM HEPES pH 7.5, 10 mM NaFl, 1 mM sodium orthovanadate, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride) along with 1 tablet of Complete miniprotease inhibitor (Amersham, Piscataway, NJ, USA). The protein concentration of lysate was determined with microBCA assay (Pierce, Rockford, IL, USA). Protein samples were separated on a 10% SDS polyacrylamide gel at 100 V for 90 min. The proteins were transferred to polyvinylidene fluoride membrane with 300 mA at 4°C for 1 hr. The membranes were blocked for 1 hr using 5% nonfat dry milk in TBSTG (20 mM Tris base, 137 mM NaCl, pH 7.2, 0.3% Tween

20, 0.5% gelatin) and incubated overnight at 4°C with primary antibodies (1:5000, in TBSTG). The membranes were washed three times for 10 min each with TBSTG before incubation with the respective HRP-conjugated secondary antibodies (1:2000, in TBSTG) for 1 hr at RT. After three washes, the protein bands were visualized with enhanced chemiluminescence method (ECL plus, Amersham). The membranes were scanned with Intelligent Box II (Fugifilm, Tokyo, Japan) and LAS-1000 Lite image analysis software (Fugifilm). Optical density of the bands was calculated with Image Gauge 4.0 (Fugifilm). The band density were normalized relative to controls and presented as mean ± SD. The results were reported as a percent of control (n=5).

8. Statistical method

Data are expressed as mean ± SD of five individual monolayers. Differences between mean values were tested for the significance using Student *t* test ($P < 0.05$).

III. Results

1. Cytotoxicity of alpha-tocopherol

AlamarBlue assay revealed that no alpha-tocopherol toxicity was observed for 24 hr up to 100-fold physiological serum level.

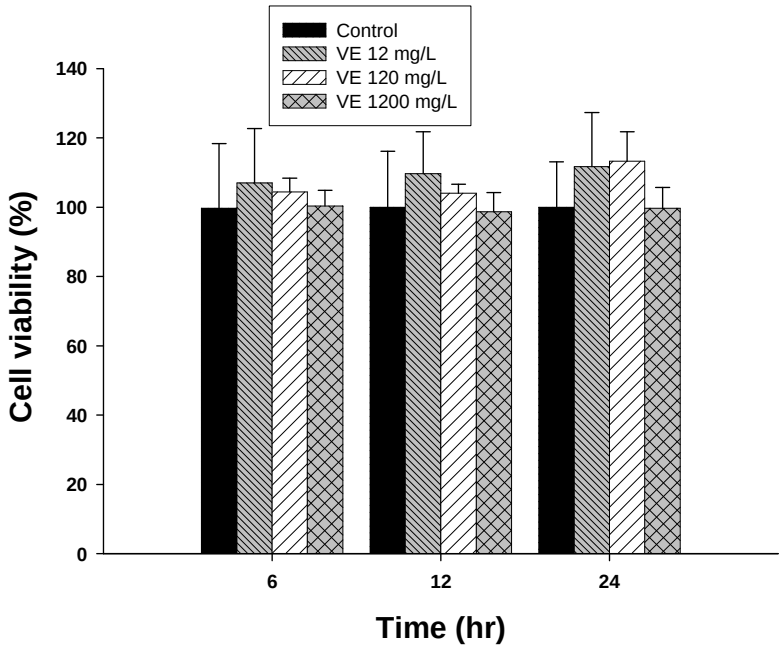

Fig. 1. Percentage of cell viability of rat brain microvessel endothelial cells after alpha - tocopherol (VE) treatment. The cell viability was assessed using AlamarBlue assay. Treatment of alpha-tocopherol up to 1200 mg/L shows no significant decrease in viability when compared with control level. Each groups are added with ethanol (final concentration 0.01 %). Data are shown as mean ± SD (n=5). ($P < 0.05$).

2. Effect of alpha-tocopherol pretreatment on cell viability

The preincubation of physiological level (12mg/L) of alpha-tocopherol efficiently suppressed H_2O_2-induced cytotoxicity. Cell viability began to decrease with 1 hr treatment of 0.2 mM H_2O_2.

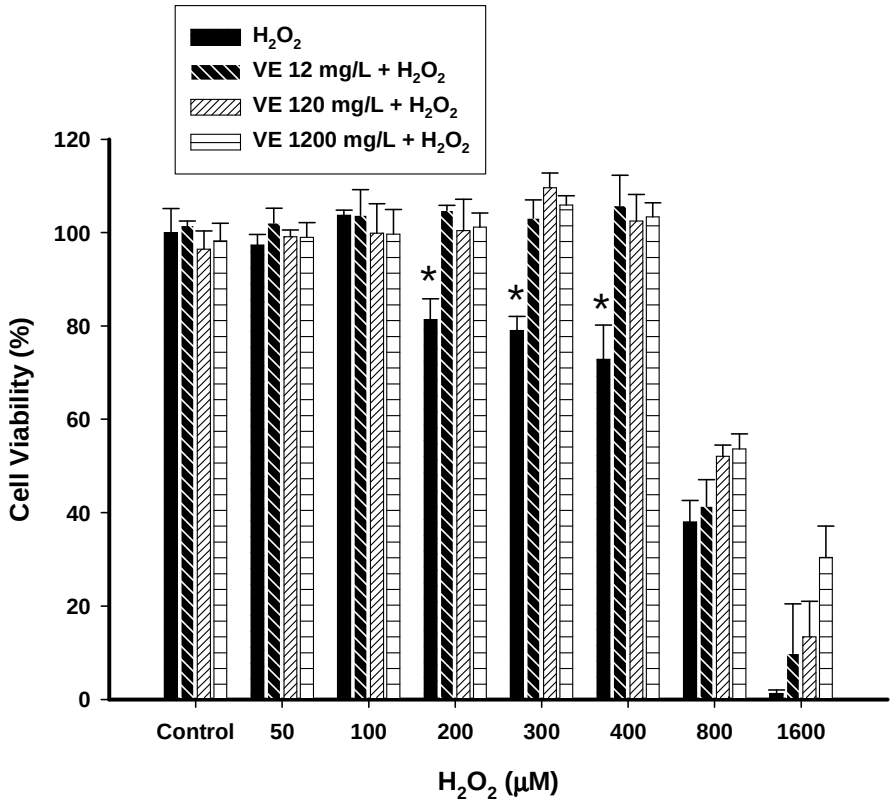

Fig. 2. Percentage of cell viability of rat brain microvessel endothelial cells with 24 hr preincubation of alpha-tocopherol (VE) and followed H_2O_2 treatment for 1 hr. The cell viability was assessed using AlamarBlue assay. Preincubation of alpha-tocopherol of 12 mg/L efficiently suppresses the decrease of cell viability (*) when compared to control. Data are shown as mean ± SD (n=5). (* : $P < 0.05$).

3. Phase contrast microscopy

Incubating RBMECs in the presence of alpha-tocopherol (12mg/L) did not cause any observable cellular damage (Fig. 3AB). To evaluate the effect of alpha-tocopherol, RBMECs were preincubated with alpha-tocopherol (12mg/L) for 24 hr, and followed by treatment of 0.3 mM H_2O_2 for 1hr. 0.3 mM H_2O_2 resulted in a significant cellular injuries (Fig. 3C), but the alpha-tocopherol suppressed H_2O_2-induced injury (Fig. 3D).

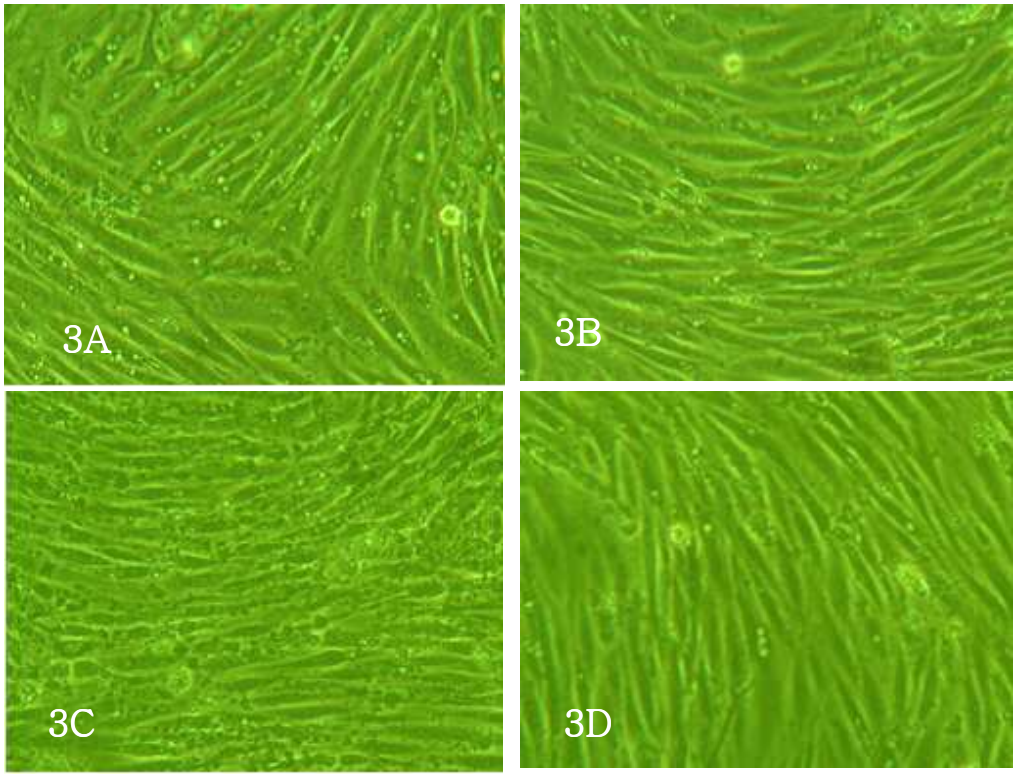

Fig. 3. Phase contrast observations (※200) of the H_2O_2-damaged RBMECs preincubated with or without alpha-tocopherol (12mg/L). (A) nontreated control; (B) preincubation with alpha-tocopherol for 24 hr; (C) 0.3 mM H_2O_2 treated for 1 hr; (D) 0.3 mM H_2O_2 treated after preincubation of alpha-tocopherol for 24 hr.

4. Confocal microscopy of RBMEC monolayer

The level of immunoreaction of TJ occludin in control (Fig. 4A) was not different from alpha-tocopherol (12mg/L) treated group (Fig. 4B). Occludin decreased when treated with 0.3 mM H_2O_2 (Fig. 4C). Occludin did not decrease by 0.3 mM H_2O_2 in alpha-tocopherol (12mg/L) preincubated group (Fig 4D).

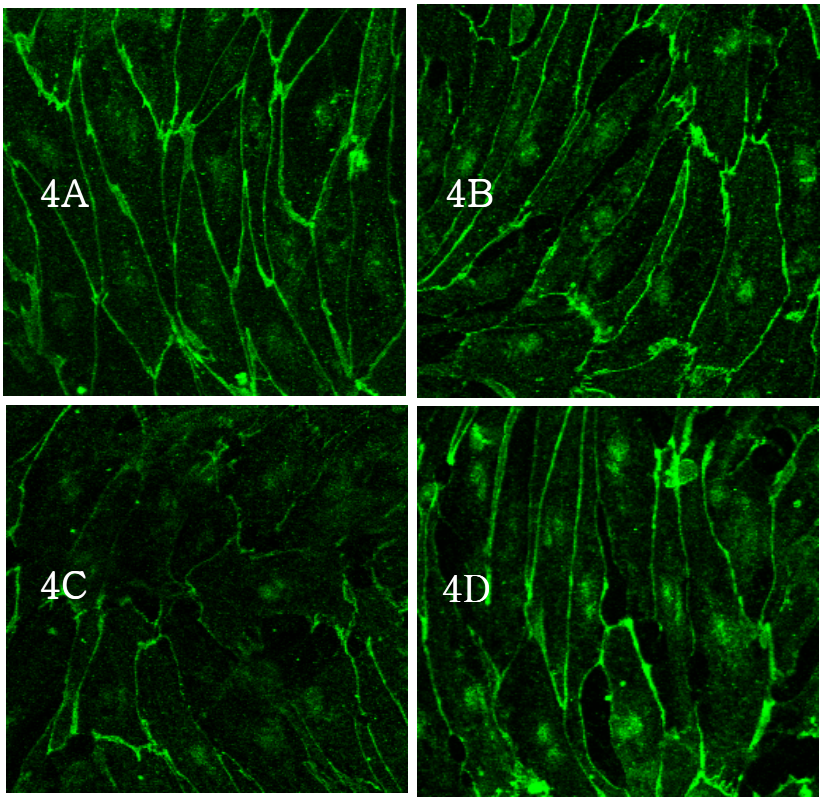

Fig. 4. Immunofluorescence staining showing occludin localization in rat brain microvessel endothelial cells. A shows occludin immunostaining in control. B shows occludin immunostaining after 24 hr pretreatment of alpha-tocopherol (12mg/L). C exhibits decreased occludin immunostaining after 1 hr exposure of 0.3 mM H_2O_2. D shows occludin immunostaining with 24 hr pretreatment of alpha-tocopherol (12mg/L) followed by 1 hr exposure of 0.3 mM H_2O_2. Note that H_2O_2 causes a loss of occludin at tight junctions in only H_2O_2 treatment group (C).

5. Western blot analysis of occludin

By immunoblotting, alterations in expression of TJ occludin that form TJs and were examined. Alpha-tocopherol did not change the level of occludin (~65 kD) when compared with control (Fig. 5.) H_2O_2 significantly decreased the level of occludin. But pretreatment of alpha-tocopherol blocked the effect of H_2O_2 (Fig. 5).

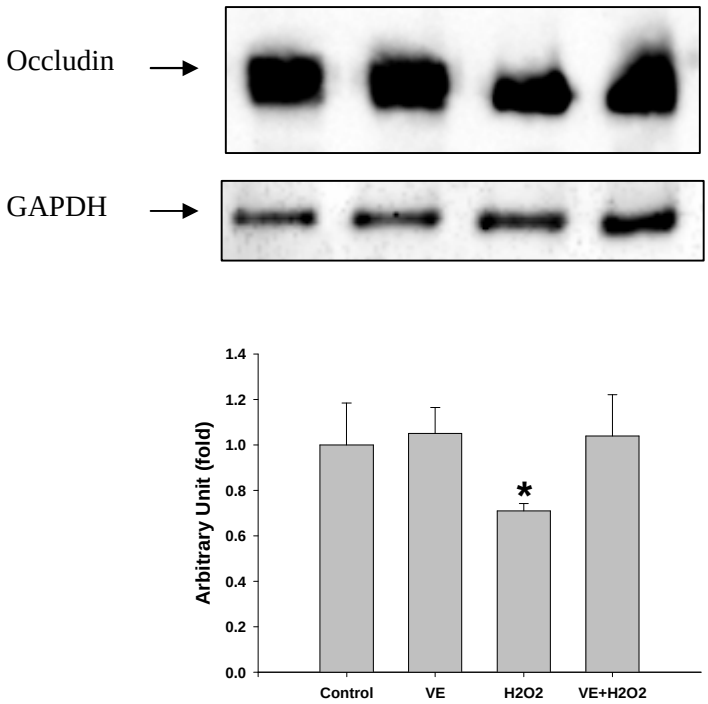

Fig. 5. Effect of alpha-tocopherol and H_2O_2 on occludin levels in rat brain microvessel endothelial cells. Occludin is not changed in control and alpha-tocopherol (VE, 12mg/L) pretreated group for 24 hr. Occludin decreases after 1 hr exposure of 0.3 mM H_2O_2. Pretreatment of alpha-tocopherol (12mg/L) for 24 hr sufficiently block the effect of 1hr exposure of 0.3 mM H_2O_2. Data are shown as the mean ± SD with * P < 0.05 (n=5).

IV. Discussion

The tight junctional protein, occludin, plays an important role in maintaining endothelial solute barriers in CNS[23,24]. Several studies have shown that H_2O_2 is involved in permeability change of tight junctions in various cells[25,26]. There is no known protective effect of alpha-tocopherol on occludin of tight junction in BBB. In the present study, we demonstrated the beneficial effect of alpha-tocopherol on oxidative stress by using primary cultures of RBMECs.

Exposure to alpha-tocopherol alone upto 1.2 g/L (2.8 mM) for 24 hr did not influence cell viability. In the second set of experiments, we examined the protective effect of alpha-tocopherol on H_2O_2-induced cytotoxicity by AlamaBlue assay. H_2O_2 concentration of 0.1 mM and below did not affect cell viability. Exposure of the RBMECs cultures to H_2O_2 (0.2–0.4 mM) for 1hr reduced cell viability in a concentration-dependent manner. Preincubation of RBMECs with alpha-tocopherol (0.028–2.8 mM) for 24 hr suppressed H_2O_2-induced cytotoxicity. But alpha-tocopherol did not protect RBMECs from more than 0.8 mM H_2O_2. With these results, the H_2O_2 concentrations between 0.2 and 0.4 mM were used in following studies. The cell morphology was studied using phase contrast microscope revealed that preincubation with alpha-tocopherol suppressed H_2O_2-induced RBMECs injury.

In confocal laser microscopy, occludin normally has a continuous distributing pattern at cell-to-cell contact boundaries. H_2O_2 caused decreased immunoreaction and loss of occludin at TJs. The loss of TJ occludin at cell-to-cell contact sites correlate

with increased paracellular permeability[27,28]. The preincubation of alpha-tocopherol suppressed H_2O_2-induced loss of occludin at TJs. Western blot also revealed that preincubation of alpha-tocopherol prevented the loss of occludin.

Occludin with a molecular mass of ~65 kD has NH_2 and COOH termini in the cytoplasm with two extracellular loops projecting into paracellular space[29]. ZO-1, ZO-2 and ZO-3 are membrane-associated guanylate kinase-like homologues (MAGUKs). To date, three MAGUKs, ZO-1, ZO-2, and ZO-3, have been identified as component of TJs[30]. Members of this family are often found at the site of cell-to-cell contact and may function to couple extracellular signaling pathways with the cytoskeleton like actin[31].

Indeed, ROSs are generated by specialized plasma membrane oxidases or by mitochondria in response to various growth factors or cytokines[32]. ROSs have been traditionally regarded as toxic byproduct of metabolism, and cells develop several antioxidant enzymes to protect themselves from these toxic species. However, recent data suggest that ROSs are also essential participants in signaling[33,34]. Besides the activation of different members of signaling cascades, ROSs may directly regulate the activity of transcription factors[35,36,37].

Vitamin E is an essential micronutrient involved in various processes relevant to human health and disease. Vitamin E is the generic term for a series of tocopherols and tocotrienols. Tocopherols and tocotrienols are further separated into individual compounds. Each of these forms of vitamin E has a different biopotency. Alpha-tocopherol is generally considered to be the most important form[38,39]. However, the

functions of tocotrienols have not been fully elucidated.

Vitamin E consumption did not alter results on any of the tests of hepatic or renal function, hematologic status, plasma lipid or lipoprotein concentrations, bleeding time, serum autoantibody concentrations, or the ability of neutrophils to kill *Candida albicans*[40]. Ingestion of vitamin E at dosages of 100–3200 IU/day for periods of 4 week to a few months was not associated with any adverse effects[41]. Human subjects were given 2000 IU vitamin E/day for periods up to 36 months in the DATATOP study without adverse effects[42]. Mean serum alpha-tocopherol concentration were about 12 mg/L or 28 μM[43]. The recommended dietary allowance for alpha-tocopherol is 15 mg/day. The no observed adverse effect level of vitamin E, a dose which produces no adverse effects as determined by the U.S. Council for Responsible Nutrition is 800 mg (1,200 IU).

Vitamin E might slow functional deterioration of Alzheimer disease with 2000 IU/day for 3 years[44]. It was proposed that chronic, high dose vitamin E dietary supplementation or parenteral vitamin E administration might serve as a successful therapeutic strategy for the prevention or treatment of Parkinson disease[45]. Even though the efficacy of vitamin E in the management of cardiovascular disease has been shown[46], the potential role of vitamin E in the treatment of cerebrovascular disease remains essentially unknown.

Although vitamin E has long been considered just as an antioxidant, it has now become clear that vitamin E has functions far exceeding that as an antioxidant. These include regulation of cellular signaling processes and gene expression. Alpha-

tocopherol induced cytochrome P450 enzyme (CYP) Cyp3a11, the murine homolog to human CYP3A4. CYPs were induced via the activation of the pregnane-X-receptor (PXR), a member of the family of nuclear receptors. Vitamin E induced a reporter gene driven by PXR. These findings revealed that vitamin E was able to directly influence gene activity[47].

The present findings provide evidence that alpha-tocopherol may be beneficial for cellular protection from ROS-mediated diseases. Since alpha-tocopherol was demonstrated to have far fewer adverse effects, it would become a noteworthy nutrient or drug for treatment of neurodegenerative diseases. Further studies are needed to elucidate how alpha-tocopherol influences gene activity in response to various stresses. Therapeutic strategies aimed at controlling endothelial cell function by intervening ROS mediated cell responses have widely applicability in vessel involved diseases.

V. Conclusions

Vitamin E is the most important lipid-soluble antioxidant in humans. Although α-tocopherol is suggested that it has protective effect from many diseases, little is known about prevention of occludin alteration under pathologic insults producing ROSs. In this study, the effects of α-tocopherol on H_2O_2-induced TJ occludin were studied with primary culture of rat brain microvessel endothelial cells. α-tocopherol had no apparent cytotoxicity upto 2.8 mM. The preincubation with α-tocopherol suppressed H_2O_2-induced cytotoxicity in AlamarBlue assay and phase contrast microscopy. In confocal laser microscopy and western blot, H_2O_2-induced loss of occludin was suppressed by preincubation with α-tocopherol. The present findings provide evidence that α-tocopherol may have a therapeutic significance for cellular protection from pathologic insults. Since α-tocopherol was demonstrated to have far fewer adverse effects, it would become a noteworthy nutrient or drug for treatment of neurodegenerative diseases. Further studies are needed to elucidate how α-tocopherol mediates the suppression of ROS-induced TJ disfunctions in BBB. Therapeutic strategies aimed at controlling endothelial cell function by intervening ROS mediated signaling cascades have widely applicability in vessel involved diseases.

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ABSTRACT(IN KOREAN)

과산화수소에 의한 치밀이음부 Occludin의 변화에 대한 α-tocopherol의 보호 효과

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비타민 E는 지용성 항산화제로 널리 알려져 있다. 그 중 α-tocopherol 은 부작용이 거의 없어 노화에 따른 여러 질환에서 영양소나 치료제로서 주목 받고 있다. 많은 질환에서 α-tocopherol 의 보호 효과가 알려져 있지만 활성 산소종이 발생되는 병적인 조건에서 혈액뇌장벽의 치밀이음부 occludin 에 대한 효과는 거의 알려져 있지 않다. 본 연구에서는 쥐의 뇌 미세혈관 내피세포를 일차 배양하여 α-tocopherol 이 과산화수소에 의한 치밀이음부 occludin 의 변화를 보호할 수 있는지를 관찰하였다. α-tocopherol 은 매우 높은 농도 (2.8 mM) 에서도 세포 독성이 나타나지 않았다. AlamarBlue assay와 위상차 현미경을 통해 관찰한 결과, α-tocopherol 을 전처치 하면 과산화수소에 의한

세포독성이 억제되었다. 공초점현미경과 western blot 을 통해 관찰한 결과, 과산화수소에 의한 occludin 의 소실은 α-tocopherol 을 전처치하여 막을 수 있었다. 이 결과는 α-tocopherol 이 여러 질환으로부터 뇌 미세혈관 내피세포를 보호하는 효과를 나타내며 α-tocopherol 이 부작용이 거의 없으므로 퇴행성 신경질환의 치료에 이용될 수 있음을 제시하고 있다. 앞으로의 연구에서는 혈액뇌장벽에서 활성산소종에 의한 치밀이음부 occludin 의 변화를 억제하는 α-tocopherol 의 기전을 밝혀야 할 것이다.

핵심되는 말 : α-tocopherol, 혈액뇌장벽, 치밀이음부, occludin, 과산화수소