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Antioxidants of new compounds from marine Algae prevent cell death of endothelial cells

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Cytosolic oxidation by 4-hydroxy-2-nonenal (4HNE) and tert-butyl hydroperoxide (t-BHP) results in cell death of bovine aortic endothelial cells (BAEC). In this study, we have investigated the roles of antioxidants such as 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether (TDB) and phloroglucinol in preventing cell death. After treatment with oxidants for 6h, cells became compact and showed nuclear condensation, which were characteristics of early apoptosis. After 12h treatment, morphologic features including severe cytoplasm condensation, membrane blebbing, and apoptotic bodies were prominent and these findings were interpreted as characteristics of late-apoptosis. When the apoptotic cells were treated with antioxidants for 12h, both early and late apoptotic cells did show no significant change. After oxidant treated cells were incubated with antioxidant for 24h, the characteristics of early-apoptosis were eliminated but cells in late-apoptosis could not return to normal cells. These results suggest that TDB and phloroglucinol prevent the cells from dying through apoptosis induced by 4HNE and t-BHP in early stage.

Key words : Apoptosis, 4-hydroxy-2-nonenal, Tert-butyl hydroperoxide, Antioxidants

INTRODUCTION

Apoptosis is a distinctive form of cell death designed to eliminate unwanted cells through activation of a coordinated and highly regulated process (Foresti *et al.*, 1999). It is involved in numerous physiologic and pathologic conditions such as immune reactions, cerebrovascular disease and Alzheimer's disease (Bankson *et al.*, 1993). The cells undergoing apoptosis show numerous morphologic changes. Initially apoptotic cells display nuclear chromatin condensation and fragmentation. Then the formation of cytoplasmic blebs and apoptotic bodies can be seen, which is followed by phagocytosis of apoptotic cells or bodies (Kerr *et al.*, 1972 ; Nagata, 2000 ; Wyllie *et al.*, 1980). Under various physiologic process and pathologic condition, apoptosis can be activated by a variety of death-triggering signals, ranging from the lack of growth factor to specific injurious agents, such as cancer drugs and radiation (Hahn *et al.*, 1994 ; Riley, 1994 ; Warren *et al.*, 2000). Oxidative stress has been described to be a major inducer of apoptosis (Forrest *et al.*, 1994).

To study the relationship between oxidants and apoptotic change, 4-hydroxy-2-nonenal (4HNE) and tert-butyl hydroperoxide (t-BHP) were used as oxidants producing oxidative stress. Vascular

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endothelial cells are important structural and functional components in all tissues and constantly subjected to oxidative stress and the attack of free radicals. So vasocular endothelial cells were used in deducing the relationship between oxidative stress and apoptosis in the present study. We have investigated whether antioxidants, 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether (TDB) and phloroglucinol extracted from *Symphyocladia latiuscula* and *Ecklonia stolonifera*, could prevent the cells from dying through apoptosis by 4HNE and t-BHP or not.

MATERIALS AND METHODS

Cell culture

Bovine aortic endothelial cells (BAEC) were provided by Dr. Kuwano at Kyushu University, Japan. The cells were cultured in Dulbecco's Modified Eagle medium (DMEM, GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, GIBCO BRL) in a humidified incubator at 37℃ and 5% CO_2.

Cell treatment with antioxidant and/or oxidant

4HNE and t-BHP were purchased from Sigma Chemical Co. (St Louis, MO), 2,3,6,-tribromo-4,5-dihydroxy benzyl methyl ether (TDB) and phloroglucinol were provided by Dr. J. S. Choi at Pukyoung National University (Busan, Korea). All agents were diluted with media, and stored at under -20℃. Cells were subcultured and incubated for 24h. 0.2 μM 4HNE or 10μM t-BHP was applied to the cells for 6h or 12h. Then oxidant was removed, cells were rinsed and further incubated without oxidant and harvested or stained after 12h or 24h; or antioxidant of 100 μM TDB or 150 μM phloroglucinol were added to the oxidant-treated cells for 12h or 24h prior harvesting or staining.

Determination of cell viability

Cell viability was determined at the indicated times by the trypan blue exclusion method. Each cell suspension (0.1$m\ell$) was mixed well with 0.1$m\ell$ of 0.2% trypan blue solution. After 2 min, each solution was placed on a hemocytometer, the blue-stained cells were counted as nonviable ones. The viability percentage was calculated based on the percentage of unstained cells (Sandoval *et al.*, 1997).

Assessment of changes in cellular morphology

The cells were plated in 35㎜ Acas dishes (Mat-Tek, USA). Each cell group was stained with 100μg /$m\ell$ acridine orange (Sigma Chemical Co.) and 100 $\mu g/m\ell$ ethidium bromide (Sigma Chemical Co.). Stained cells were visualized under a reflected fluorescence microscopy (Olympus, Japan). Under fluorescence microscopy, green colored cells were interpreted as living ones. Apoptotic cells displayed red color. The examination was performed with a laser cytometry (ACAS 570). In observation with laser cytometry, living portion of the cells exhibited purple and blue color and dying or dead cells revealed red color.

Assessment of changes in cellular morphology at ultrastructural level

The cells were grown in 6-well plate and were harvested with cell scraper. Cell suspension was centrifuged and cell pellet fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 12-24h, postfixed with 1% osmium tetroxide (OsO_4) for 2h, dehydrated through a series of graded alcohol solutions, and finally embedded in spur resin (Electron Microscopy Sciences Co., Ltd.). Ultrathin sections (60㎚) were cut using a LKB ultramicrotome (Nova, Swededn) and double stained with lead citrate and uranyl acetate. The

examination was performed with a JRM 1200 EX-II electron microscope (JEOL, Japan).

TUNEL assay

Terminal deoxynucleotidyl transferase (TdT)-mediated X-dUTP nick-end labeling (TUNEL) kit was used according to the manufacturer's instructions (Boehringer Mannheim). The cells were fixed with paraformaldehyde, Triton X-100 permeabilized, then incubated with a TUNEL reaction mixture containing fluorescein-dUTP and TdT to catalyze attachment of fluoresceinated dUTP to the free 3'-OH ends of DNA strand breaks. Then cells incubated with Converter-Peroxidase (POD), incubated with DAB-substrate solution, and finally covered with a slide glass. The slides were examined by light microscopy.

DCFDA assay

O_2^- scavening activity was examined by using DCFDA assay capable of detecting oxygen free radical. 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA ; f.c., 2.5μM) was incubated at 22℃ for 10 min and placed on ice in the dark room until immediately prior to the study. H_2DCFDA is deacetylated to non-fluorescent 2,7-dichlorodihydrofluorescein (DCFH) by esterase and subsequently oxidized to highly fluorescent 2,7-dichlorofluorescein (DCF) by O_2^-. The fluorescence intensity of oxidized DCFH was measured by a microplate fluorescence reader (FL 500, Bio-Tex instruments) at the excitation and the emission wavelengths of 485nM and 530nM, respectively for 1h.

RESULTS

Oxidants induce apoptosis in endothelial cells

The cell viability was significantly reduced in oxidant treated groups. There was little difference between effectiveness of 4HNE and one of t-BHP. The incubation period was more important factor in inducing cell death of endothelial cells by oxidants (Table 1).

Table 1. Cell viability (%) of cultured BAEC exposed to oxidants

Samples	Hours of treatment	
	6h	12h
Control	100.0± 3.1	100.0± 4.2
4HNE (0.2㎛)	42.6± 3.6	10.3± 3.4
t-BHP (10㎛)	37.3± 2.4	7.2± 1.5

Values show the relative % of cell viability by trypan blue assay.

To determine that observed cell death induced by oxidants was the apoptotic cell death, we analyzed morphologic characteristics using fluorescence microscopy. The cells of the control group were normally appeared and entirely displayed green color (Fig. 1A). In the cells treated with oxidants for 6h, cytoplasmic and nuclear condensation were observed. These morphological changes means that cells are in early apoptosis stage (Fig. 1B&2B). In 12h treated cells, there were some characterstics of late-apoptosis that included further cell condensation, formation of membrane vesicle (apoptotic bodies) and membrane blebbing (Fig. 1C&2C). The colors of the cells treated with oxidant for 6h or 12h were red. These findings have indicated that both cytoplasm and nucleus were in apoptotic state.

Under laser cytometry, the cells of the control group showed purple and blue. The oxidant-treated cells showed gradually green, yellow, and red color, regardlesss of incubation time (Fig. 3A-C).

To observe the apoptotic change more definitely, characteristics of apoptosis were ultra-structurally observed in cells treated with oxidant for 6h or 12h. Especially, findings of cell condensation, formation

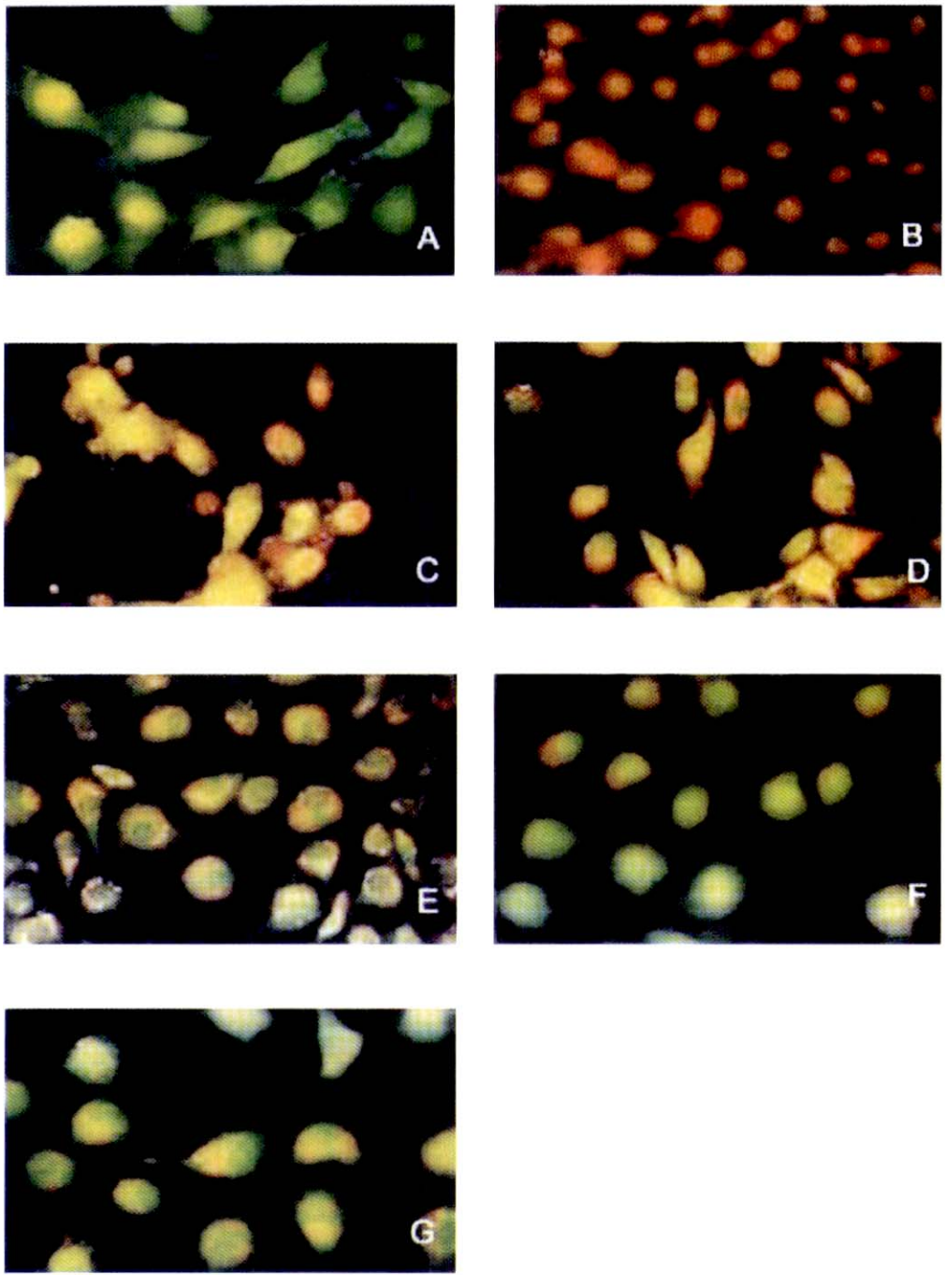

Fig. 1. Recovery of 4HNE-treated BAEC by phloroglucinol (PG) using fluorescence microscopy.
(A) Control (NO treatment), (B) 4HNE-6h, (C) 4HNE-12h,
(D) 4HNE-6h + PG-12h, (E) 4HNE-12h + PG-12h,
(F) 4HNE-6h + PG-24h, (G) 4HNE-12h + PG-24h.

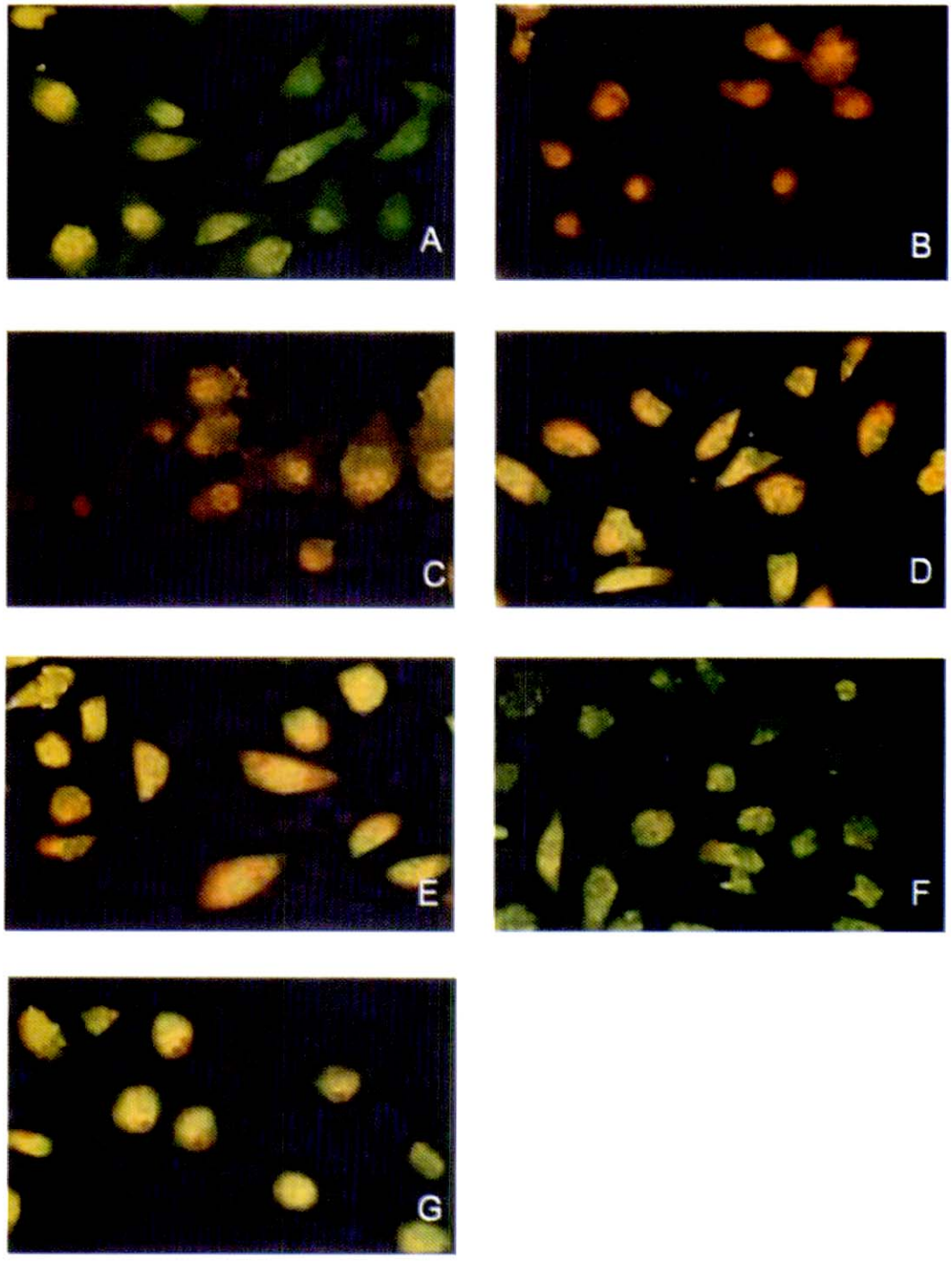

Fig. 2. Recovery of t-BHP-treated BAEC by TDB using fluorescence microscopy.

(A) Control (NO treatment), (B) t-BHP-6h, (C) t-BHP-12h,

(D) t-BHP-6h + PG-12h, (E) t-BHP-12h + PG-12h,

(F) t-BHP-6h + PG-24h, (G) t-BHP-12h + PG-24h.

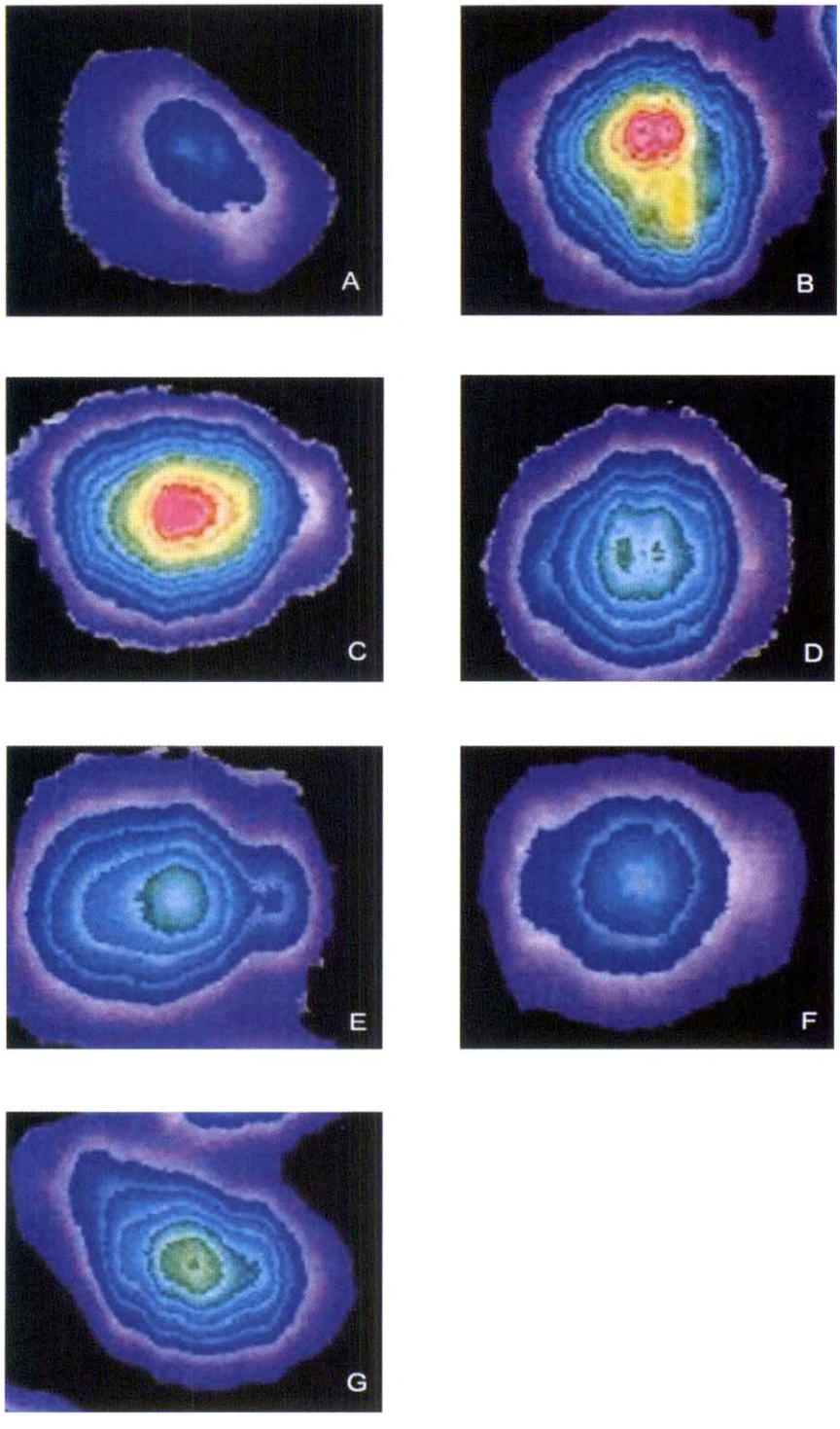

Fig. 3. Recovery of 4HNE-treated BAEC by phloroglucinol (PG) using laser cytometry.
(A) Control (NO treatment), (B) 4HNE-6h, (C) 4HNE-12h,
(D) 4HNE-6h + PG-12h, (E) 4HNE-12h + PG-12h,
(F) 4HNE-6h + PG-24h, (G) 4HNE-12h + PG-24h.

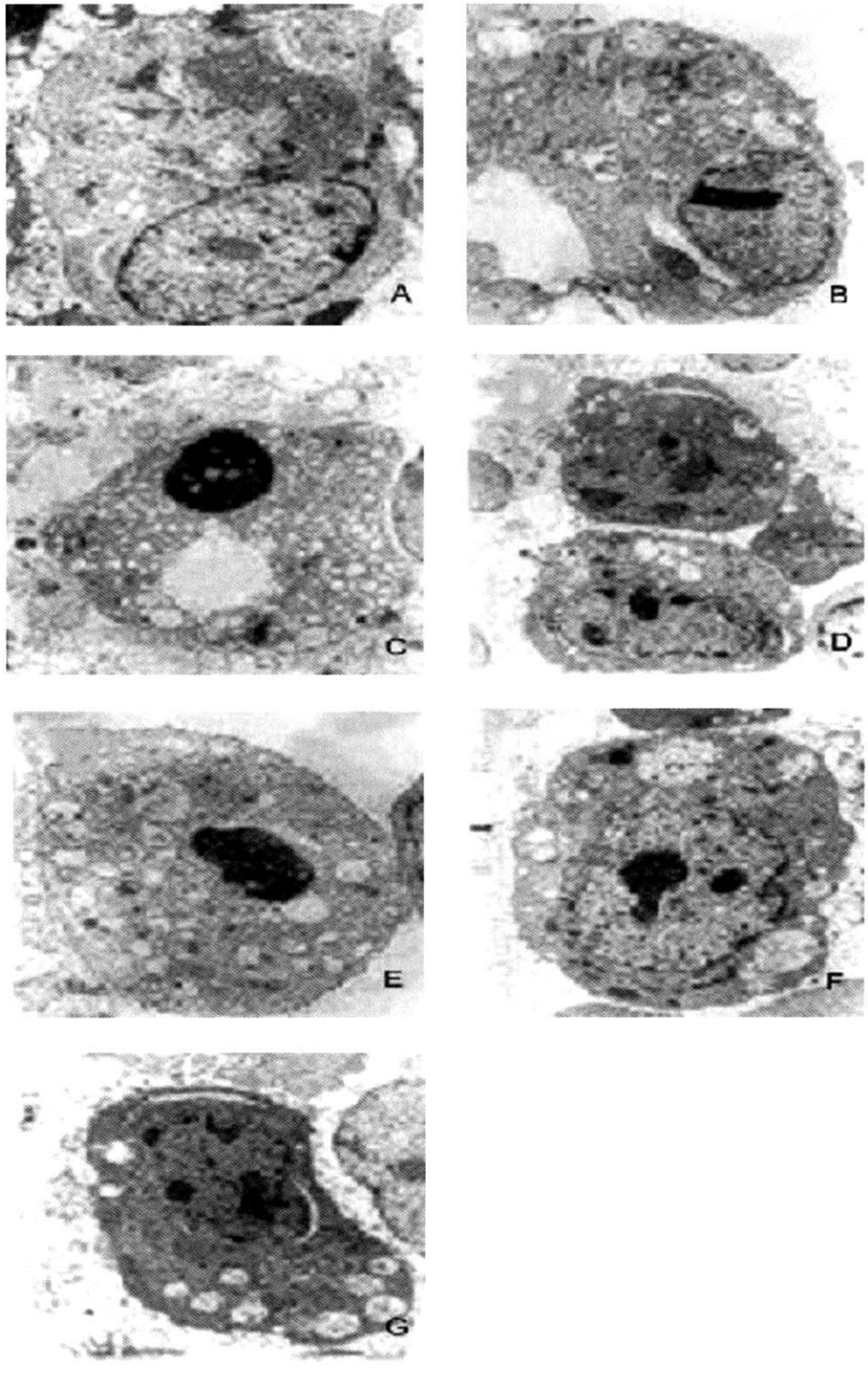

Fig. 4. Recovery of apotosis in t-BHP-treated BAEC by TDB using transmission electron microscopy.
(A) Control (NO treatment), (B) t-BHP-6h, (C) t-BHP-12h,
(D) t-BHP-6h + PG-12h, (E) t-BHP-12h + PG-12h,
(F) t-BHP-6h + PG-24h, (G) t-BHP-12h + PG-24h.

of apoptotic bodies, membrane blebbing and cell shrinkage were prominent. (Fig. 4A-C).

The TUNEL assay was investigated to immunologically detect the apoptosis of the individual cells. The treated cells exhibited brown color, especially in nucleus (Fig. 5). The present study shows that type of cell death induced by 4-HNE or t-BHP is apoptosis, not necrosis.

TDB & PG can prevent cells from dying through apoptosis

One thirds to half of cell portion recovered to normal status in the 12h antioxidant treatment group. The nuclei of the cells were green-colored, even though the cytoplasm have red area (Fig. 1D&E, 2D&E). When the damaged cells were treated with antioxidants for 24 h, most of the cell portion were in green color in the cells treated with oxidant for 6h (Fig. 1F&2F). This findings suggest that the cells in early apoptotic state recovered to ones in normal state. But the cells treated with oxidant for 12h still exhibited red color in a certain area (Fig. 1G&2G). This means the cells were not completely recovered from its apoptotic damage. There was no difference in the degree of recovery by the kind of antioxidants.

Under laser cytometry, the cells treated with antioxidants for 12h were mixed with yellow, blue and green color (Fig. 3D&E). The cells treated with antioxidants for 24h exhibited purple to green color (Fig. 3F&G). Especially cells pretreated with oxidant for 6h was entirely composed of blue and purple color, which is a characteristic of normal cell.

Recovery of cells in early apoptotic state to normal state was also ultrastruturally confirmed, though cells in late apoptosis maintained the morphologic characteristics of apoptotic damage (Fig. 4F&G).

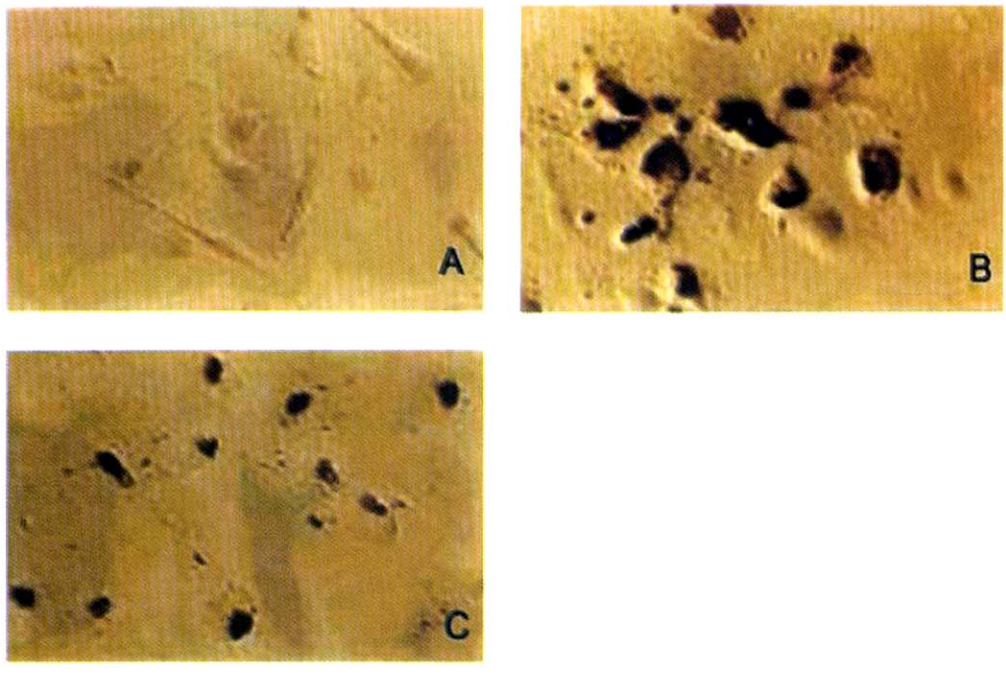

Fig. 5. Apoptosis in oxidants-treated BAEC using TUNEL assay.
(A) Control (NO treatment), (B) 4HNE-12h, (C) t-BHP-12h.

Measurement O_2^- scavenging activity using DCFDA assay

To observe and confirm the O_2^- scavenging activity of TDB and phloroglucinol, DCFDA assay was done and trolox, which has been known as standard oxidant, was used as control agent. As shown in Fig. 6, the effectiveness of TDB and phloroglucinol were different in accordance with the type of oxidant used. Even though there was difference in activity according to efficient concentration and used oxidant, the results have shown TDB and phloroglucinol could be used as effective antioxidant agent.

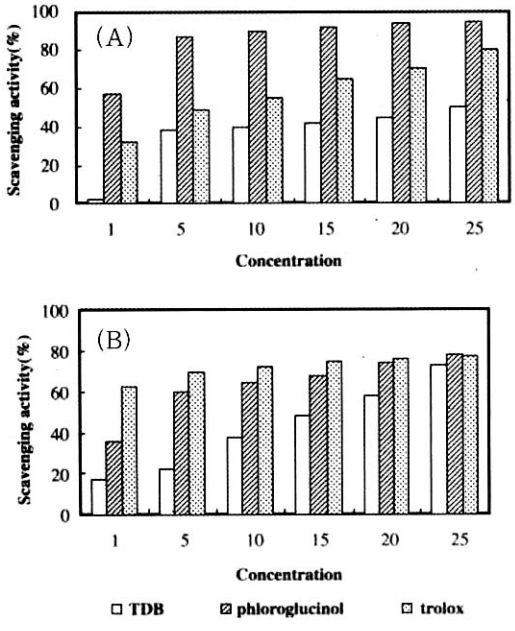

Fig. 6. O_2^- scavenging activity of TDB, phloroglucinol and trolox to 4HNE (A) and t-BHP (B).

DISCUSSIONS

Oxidative free radicals are known to cause peroxidation of polyunsaturated fatty acids in membrane. This lipid peroxidation causes the formation of reactive aldehydes (Benedetti *et al.*, 1979; 1980; Benedetti and Comporti, 1987; Li *et al.*, 1996). These aldehydes have longer biological half-lives than free radicals and can diffuse from their site of formation to distant targets and cause cellular damage, mainly in the form of apoptosis. 4HNE and t-BHP used in this study have been known as oxidant producing oxidative stress. 4HNE is a nine-carbon α,β-unsaturated aldehyde generated from β-scission of hydroperoxides of polyunsaturated fatty acids such as arachidonic and linoleic acid. 4HNE cause rapid cell death associated with deflection of sulfhydryl groups, disturbances in calcium homeostasis, inhibition of key metabolic enzymes, protein and DNA synthesis (Esterbauer *et al.*, 1991). t-BHP produce free radicals such as t-bytoxyl, t-butyl peroxyl and methyl radical, which are formed by metabolizing cytochrome C or cytochrome C_1 of mitochondria. They cause damage of lipid peroxidation, protein oxidation, and nucleic acid (Fraga and Tappel, 1988) resulting in apoptosis. t-BHP has been shown to depolarize mitochondrial membrane by increasing Ca^{2+} concentration (Forman *et al.*, 1987) and damage to cell membrane by oxygen-based radical through translocation and activation of protein kinase C (PKC). Both 4HNE and t-BHP have been associated with the etiology of aging and numerous pathologic conditions such as many ischemia/reperfusion, atherosclerosis, shock, Parkinson's disease, and Alzheimer's disease (Lovell *et al.*, 1997). *In vitro* studies have revealed that 4HNE of 1-100μM concentration induce apoptosis in non-neuronal cells and neurons (Esterbauer *et al.*, 1991; Mark *et al.*, 1997). It has been reproted that t-BHP induced apoptosis in a concentration-dependent manner over the concentration range of 0.2 to 3 mM in cortical slices (Kim *et al.*, 1998). We could observe that cell viability was hampered by 4HNE of 0.2μM concentration and t-BHP of 10 μM. The present study shows that the oxidant had strong cytotoxic effect at lower concentration, in

comparison with the need of higher concentration for inducing cell death. The authors suspect this difference is caused by difference in cell type, and suggest that endothelial cells are more susceptible to oxidative stress and rapidly progress to death by oxidants.

Using fluorescence microscopy, laser cytometry and TUNEL assay, cell death by apoptosis was confirmed. The cells treated with oxidants showed little difference in the degree of apoptosis due to oxidant type. The proportion and degree of apoptotic change was determined by exposure time to oxidant.

TDB and phloroglucinol were extracted from algae, *Symphyocladia latiuscula* and *Ecklonia Stolonifera*, respectively. TDB and phloroglucinol were shown to have effects as antioxidants (Park *et al.*, 1999). In this context, it is important to bring to notice our observations concerning effect of TDB and phloroglucinol on recovery from apoptosis. Even though cells in late apoptosis did not recovered from its state by antioxidant, apoptosis resulting in cell death was inhibited by adding the antioxidants. These results suggest that antioxidants can delay cell death by inhibiting progression of apoptotic process.

In conclusion, oxidative stress to vascular endothelial cells play an important role in development and progression of numerous degenerative diseasess and antioxidant such as TDB and pluroglucinol can block or eliminate the oxidative damage to endothelial cells. Further study is needed to utilize the antioxidant as tools to prevent and treat the degenerative disorders and aging.

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