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Protective Effects of Natural Phytochemicals on the Lipid Peroxides Induced Apoptosis in the Human Endothelial ECV 304 Cells

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Abstract: The final bio-metabolites of lipid peroxidation (LPO) such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) have been suggested to mediate the oxidative stress-linked pathological incidences. Natural phytochemicals such as polyphenolic compounds in green tea have been known in preventing the LPO induced cellular growth inhibition and apoptosis. We investigated that green tea ethanol extracts (GTE) inhibit LPO-induced apoptosis in ECV 304 cells. GTE had time- or dose-dependent anti-apoptotic effects as evidenced by changes in cell morphology, MTT assay, DNA fragmentation, LPO production, and the Western blotting for apoptotic expression. In the 4-HNE-induced apoptosis model, GTE 10-20 μg/mL decreased cell death through decreasing LPO production. GTE protected 4-HNE induced apoptosis, as evidence with down regulation of mitochondrial signaling such as cytochrome C and caspase-3 activity. GTE increased bcl2, survival signaling protein, compared to 4-HNE alone within 6 hr incubation. Since polyphenols in GTE are effective antioxidants in endothelial ECV 304 cells, we suggested that natural polyphenols might be anti-atherosclerotic.

Keywords: phytochemical, 4-hydroxynonenal, mitogen-activated protein kinase, cytochrome C, apoptosis

Introduction

Novel facts that phytochemicals or polyphenolics, which have been known to be abundantly present in the natural foods until now, have various physiological activities have been of increasing interest (1). These molecules play a role as a natural herbal substance that maintains the integrity of capillaries and supports the functions of vitamin C, the natural anti-oxidant (2). They also suppress the synthesis of free radicals and then have anti-oxidative, anti-bacterial, anti-cancer, and anti-inflammatory effects, thus lowering the incidences of cardiovascular diseases and cancer (3-6). An intake of soy products (e.g., green tea, wine, soybean paste, *tofu*, and soybean oil), which have a plenty amount of phytochemicals or polyphenolics, has been reported to be effective in preventing the inflammatory vascular diseases (7,8). Green tea extract contains high level of polyphenols (a bioflavonoid), and it's also a rich source of epigallocatechin gallate (EGCG), that's over 200 times more potent than vitamin E in fighting free radicals and pro-oxidants (7,8). Quercetin and EGCG lower the synthetic rate of oxidized low density lipoprotein (OxLDL) with the ability to remove the free functional groups and thereby inhibit the proliferation of atherosclerotic plaque (9). It has also been reported that the above molecules were effective in reducing the inflammatory responses associated with allergy, having an anti-cancer effect, and decreasing low density lipoprotein (LDL) oxidation (10). In Japanese epidemiological study, the concentrations of serum cholesterol, triglyceride, and lipoproptein were lower in the elderly who drank more than 10 cups of tea a day (11,12). According to an animal experiment using rats, green tea, or tea was effective in markedly suppressing the size of tumor and the number of tumor cells (13).

4-Hydroxy-*2E*-nonenal (4-HNE) and malondialdehyde (MDA) are major peroxide products of dietary polyunsaturated fatty acids (PUFA) are a conjugated-acetaldehyde molecule with an *in vivo* toxicity (14,15). The correlation between 4-HNE and atherosclerosis has been examined by Ruef *et al.* (16) in Germany from 2001 (17). A mitogenic effect depending on the concentration of 4-HNE was observed in the vascular endothelial cells. It has also been reported that 4-HNE is involved in inducing the synthesis of NFκB, a major transcription factor for apoptosis which is sensitive to the oxidation-reduction reactions (18). Furthermore, epidermal growth factor (EGF) induced the signal transduction system for receptor cells in HECV-304, the endothelial cells lining the human aorta and the concentration of OxLDL was simultaneously elevated (19). However, there were no direct evidences that either 4-HNE or OxLDL is involved in the apoptosis (20). Moreover, few studies have examined whether a dietary-induced synthesis of 4-HNE increases OxLDL and its *in vivo* and *in vitro* effect on the apoptosis of endothelial cells (17).

Therefore, the aim of the current study is to elucidate the effect of 4-HNE or MDA on the mechanisms by which anti-oxidative and anti-apoptotic activity in the vascular endothelial cells which were priorly treated with 4-HNE or MDA, major peroxide products of dietary unsaturated fatty acids.

Materials and Methods

Cell culture and treatments Human endothelial ECV 304 cells were provided by Japanese Collection of Research

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Bioresources. ECV 304 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, San Diego, CA, USA) with 5% fetal bovine serum (FBS) and antibiotics (gentamycin sulfate) at 37°C in a humidified incubator containing 5% CO_2. To study the effects of extract fatty acids on cell growth rate, ECV 304 cells were exposed to different concentrations of fatty acids or lipid peroxides (LPO) from 1 to 48 hr. Fatty acids completely dissolved in ethanol were dried under argon gas and immediately exposed to 100% FBS, as described (21). Final concentrations of fatty acids and FBS in media were 50 μM/L and 1%, respectively. Green tea was obtained from AmorePacific Corporation (Seoul, Korea). Green tea ethanol extract (GTE) was repeatedly soaked in 70% ethanol and incubated at 45°C for 12 hr, then the extract was filtered by Whatman® paper (W&R Balston, Ltd., Kent, UK) and concentrated under the reduced pressure system. GTE were dissolved in dimethyl sulfoxide (DMSO) with final concentrations in media, 10-20 μg/mL.

Cell proliferation and toxicity Viability of ECV 304 cells after the treatment with GTE against or fatty acids such as linoleic acid (LA), docosahexaenoic acid (DHA), or arachidonic acid (AA) was determined by following the protocol supplied in the CellTiter 96 NonRadioactive Cell Viability Assay kit (Promega, Madison, WI, USA) by using (3-(4,5-dimehythiazol-2-yl)-2,5-diphenyltetrazolium) (MTT) as a substrate. ECV 304 cells were placed in 24-well plates and treated with 50 μM/L fatty acids for 12, 24, and 48 hr as described above.

Fatty acids incorporation After breaking up the ECV 304 cells, lipid was extracted using the modified Folch method (22). Methyl esters of the lipids were prepared in a chloroform/methanol/NaCl (3:47:48 by volume) solution with the methylation reagent (BF_3-methanol/methanol/benzene, 25:55:20 by volume), and an internal standard was added. The mixtures were reacted in a heating block for 90-100 min to produce methyl esters. A gas chromatograph (HP 5890 II Plus; Hewlett Packard, Palo Alto, CA, USA) was used to separate the fatty acids, which were then compared with the standard fatty acids (Sigma-Aldrich, St. Louis, MO, USA) for quantification.

LPO production Both 4-HNE and MDA derived from peroxidation of PUFA were measured as convenient indices for LPO after culturing the cells under the same conditions using LPO kit (Calbiochem Co., San Diego, CA, USA). Whole cell lysates from 3×10^6 cells were prepared by 3 repeated cycles of freeze/thaw in distilled water. Diluted samples (200 μL volume) were added into 650 μL of 10.3 mM/L *N*-methyl 2-phenylindole. Methane sulfonic acid (15.4 mol/L; final volume 150 μL) was added to mix the sample, which was then incubated at 45°C for 40 min. After cooling on ice, 4-HNE and MDA products were measured at 596 nm in comparison to the standard curves.

Caspase-3/-7 activity Using the Apo-One™ Homogeneous caspase-3/-7 assay kit from Promega (Madison), the amount of fluorescent product generated is proportional to those of caspase-3/-7 cleavage producers in the sample. ECV 304 cells were grown at a density of 2×10^5 cells/mL in each 96-well plate. After the treatment with fatty acids or LPOs for indicated times, the caspase-3/-7 reagent was added into each well of the plate at a 1:1 ratio of reagent to sample. After incubation for 5 hr at 37°C, the fluorescence of each well was determined at the excitation and emission wavelengths at 485±20 and 530±25 nm, respectively.

Western blotting for the cell signaling Whole cell extracts from LPO or fatty acids treated cells with GTE or without GTE (3×10^6 cells) were prepared in a buffer: 10 mM/L Tris-HCl, 2 mM/L ethylene diamine tetraacetic acid (EDTA), 1 μM/L leupeptin, β-mercaptoethanol, 2 mM/L EDTA, α-2-macroglobulin, 100 μM/L phenylmethylsulfonylfluoride (PMSF), and 250 mM/L sucrose. After the extracts were centrifuged (14,000×g for 20 min), the resulting supernatants were subjected to immunoblot analyses against each target protein such as mitogen-activated protein kinase (MAPK), caspase-3/-8/-9, and cytochrome C (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The conjugated 2nd antibodies against above proteins were purchased from PharMingen (San Diego, CA, USA). The β-actin was used as a control protein. Each immunoreactive antigen was visualized by using the SuperSignal West Pico ECL kit (Thermo Fisher Scientific, Rockford, IL, USA) and exposed to Kodak BioMax™ XAR film (Sigma-Aldrich) to identify the target proteins.

Statistical analysis Experimental results shown were repeated more than 3 times, unless otherwise indicated. Statistical analyses were performed using Student's *t*-test for the comparisons between 2 groups and one-way analysis of variance (ANOVA) test for the comparisons among 3 or more groups. A $p<0.05$ was considered statistically significant.

Results and Discussion

Rate of cell influx of dietary fatty acids (FA) The rate of FA influx in the cells which were treated with LA and DHA 50 μM was represented in Fig. 1. The rate of the influx of LA was approximately 2.5 times higher in the LA treatment group than that in the control group and the DHA group. The rate of the influx of DHA was 5.0 times higher in the DHA group than the LA group and the control group. These results indicate that DHA had a higher influx rate into the endothelial cells than LA. By contrast, the rate of the influx of AA (20:4, *n*6) fatty acids was 4.7 times higher in the vascular endothelial cells which were treated with LA than the control group and approximately 17 times higher than the DHA group. Based on the findings that the rate of the synthesis of LA into AA was markedly higher in the DHA group than the groups where other fatty acids were treated, the discrepancy compared to other groups can be interpreted as the effect due to the change of eicosanoid metabolism. Isolating phospholipid of plasma membrane such as phosphatidyl ethanolamine, DHA is well incorporated into cell rather than LA. These results indicate the significant values compared to the overall one (data not shown). It is therefore essential for the different tendency of the influx of fatty acids as well as the fluidity of cell membrane to isolate each phospholipid and then to examine the distribution of fatty acids.

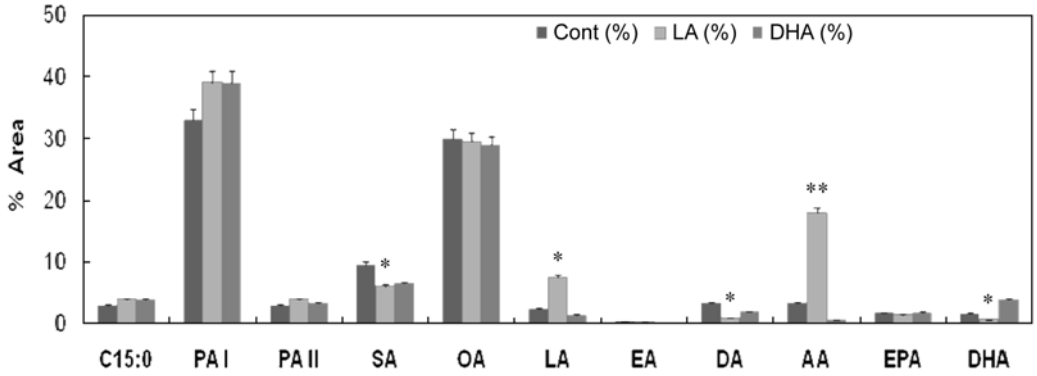

Fig. 1. The distribution of various fatty acids in EVC 304 cells originated from 50 μM of LA and DHA treatments. PA I, palmitic acid; PA II, palmitoleic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; EA, eicosaenoic acid; DA, dihomocolumbinic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. *p<0.05, **p<0.01.

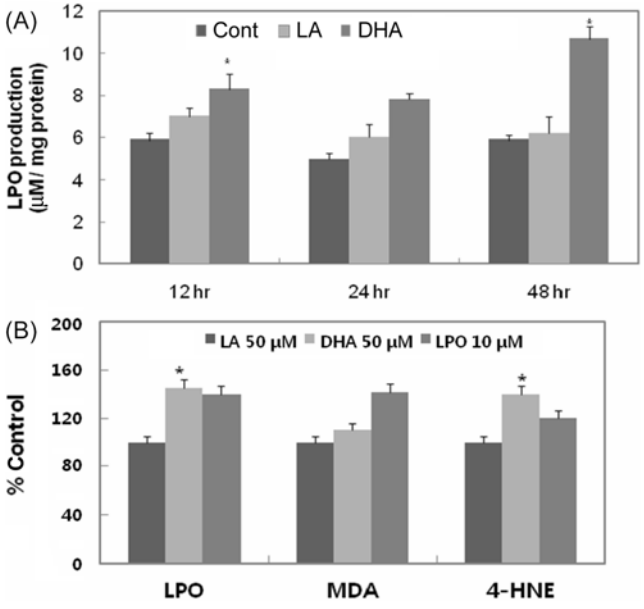

Fig. 2. Changes of LPO concentration in LA, DHA, and LPO treated ECV 304 cells. LA and DHA-induced total LPO production (μM/mg protein) according to incubation time (A) and the relative percents of the control in total LPO, MDA, and 4-HNE production (B).

Production of LPO (MDA+4-HNE) according to FA treatment The sample was treated with the representative w-6 FA, LA 50 μM, and w-3 FA, DHA 50 μM. The total amount of the resulting, final peroxide product, LPO, was significantly increased following 12 hr and 48 hr culture in the DHA group compared to LA, respectively (Fig. 2A). However, in previous study, amount of LPO following 24 and 48 hr cultivation with 50 μM of AA was increased by approximately 1.2 times compared to the LA or DHA treatment. There was a significant difference in the LPO production between the administration of dietary AA and LA even though AA was synthesized from LA *in vivo*. DHA treatment significantly (p<0.01) produced total LPO, 4-HNE and MDA, compared to LA corresponding to increasing incubation times. Even though DHA produced MDA more than 4-HNE in quantity, 4-HNE production in DHA was more differences than MDA in DHA compared to LA. (Fig 2B) LPO at 48 hr in DHA group was very similar to LPO production when 20 μM of LPO were treated for 12 hr. (data not shown) We found that the cytotoxic effect produced cell death to a significant extent following the treatment with LPO as the concentration of LPO was increased as same as in other studies (23,24). However, the cytotoxic effect of endothelial cells appeared from more than 200 μM of LPO, therefore, the trial had been performed below 100 μM. The synthesis of 4-HNE or MDA or rate of synthesis was also evaluated. This showed that the MDA/4-HNE ratio was significantly lower in the DHA group than the LA group, which indicates that the synthesis rate of 4-HNE was relatively higher. In addition, MDA did not show consistent results in ECV 304 cells as previously described in other cells. Accordingly in the current study, protective effects of ginseng against LPO were based on 4-HNE instead of MDA, a representative of LPO.

Effect of GTE on the cytotoxicity following 4-HNE treatment The sample was treated with GTE (10 μg/mL) against 4-HNE-induced ECV 304 cells at different concentrations of 50, 100, and 200 μM to examine the inhibitory effect of the cytotoxicity. As the result in Table 1, GTE decreased the cell death at all culture times up to 48 hr, but a significant difference was found at 12 hr incubation. In more detail, when 4-HNE and GTE were co-cultured for 12, 24, and 48 hr, the cytotoxic effect was suppressed at a rate of 37.5, 22.9, and 9.9% with 4-HNE 50 μM, at a rate of 21.5, 10.3, and 0.9% with 4-HNE 100 μM and at a rate of 8.5, 8.9, and 11.1% with 4-HNE 200 μM. These results indicate that GTE extract suppressed the cytotoxic effect of 4-HNE treatment but it was not effective at higher concentration of 4-HNE. Therefore, the LPO production and Western blotting were examined at higher concentration of GTE (20 μg/mL).

Inhibition of GTE on LPO production in 4-HNE induced cells The sample was treated with GTE 20 μg/mL together with 4-HNE 50 μM. This showed that the cell death was significantly decreased to a greater extent following the treatment with a 20 μg/mL of GTE than that with a 10 μg/mL of GTE even at an initial time of 1 and 3 hr. As the culture time is prolonged, however, the difference

Table 1. Differences in absorbance for MTT analysis with 10 μg/mL treatment of green tea ethanol extracts (GTE) after the different concentration of 4-HNE were treated in ECV 304 cells[1)]

	12 hr	24 hr	48 hr
Control	[2)z†]0.327±0.00[a***3)]	[z†]0.350±0.02[b***]	[z†]0.542±0.01[c***]
4-HNE 50 μM	[x†]0.271±0.01[a***]	[x†]0.253±0.00[a***]	[y†]0.361±0.02[b***]
4-HNE 50 μM+GTE	[x†]0.292±0.01[b***]	[w†]0.274±0.01[a***]	[x†]0.379±0.02[c***]
4-HNE 100 μM	[w†]0.254±0.00[b**]	[v†]0.198±0.00[a**]	[v†]0.187±0.01[a**]
4-HNE 100 μM+GTE	[y†]0.269±0.00[b*]	[y†]0.214±0.01[a*]	[y†]0.188±0.01[a*]
4-HNE 200 μM	[x†]0.174±0.03[c***]	[w†]0.151±0.01[b***]	[x†]0.088±0.01[a***]
4-HNE 200 μM+GTE	[w†]0.187±0.01[c**]	[v†]0.169±0.01[b**]	[w†]0.139±0.01[a**]

[1)]Values are the mean±SD.
[2)xyz]Means in the same column sharing different superscript are significantly different among groups ([†]$p<0.0001$).
[3)abc]Means in the same row sharing different superscript are significantly different among groups (*$p<0.05$,**$p<0.001$,***$p<0.0001$).

between GTE 10 and 20 μg/mL became negligible. Therefore, in LPO and the Western blot, 4-HNE 50 μM was treated with GTE 20 μg/mL (Fig. 3). Following a 1 hr culture of 4-HNE, LPO production was increased by approximately 3 times compared to the control group. With the concomitant treatment with GTE 20 μg/mL, however, the amount of LPO was decreased by 50%. The inhibitory effect of GTE on the synthesis of LPO showed a statistical significance until a 6 hr culture, but the concentration must be elevated if the culture is performed for a longer period of time.

The polyphenolic constituents of green tea are amphphilic substances that increase the rate of influx of plasma membrane. It was therefore assumed that a minimal amount of GTE would be sufficient for the effectiveness. This was confirmed based on the findings that the effect of GTE 10 or 20 μg/mL disappeared after 24 hr in such events as cell proliferation or LPO production.

If the activity of anti-oxidative enzymes (e.g., glutathione peroxidase) is decreased due to 4-HNE, the intracellular oxidation-reduction reactions are blocked and the resulting breakdown of signal transduction system causes a series of cascades leading to apoptosis (21,25). Under this background, our results were in agreement with the reports by Rah *et al.* (23) that GTE transmitted the cellular signals to block the oxidation-reduction reactions and polyphenolic constituents suppressed the apoptosis due to hydrogen peroxide or xanthine oxidase. Kaneko *et al.* (26) maintained that polyphenolics removed LOOH of LA which had a toxic effect in HUVEC. Above effect was particularly powerful when constituents of green tea, EGCG monoglucosid and EGCG diglucoside catechin were treated (27). Furthermore, as other mechanisms by which 4-HNE of polyphenolics is removed, its role for supply of NADPH and glutathione associated with pentose phosphate pathway must also be considered. It has been reported that quercetin as well as EGCG not only inhibits the synthesis of OxLDL associated with the pathogenesis of atherosclerosis but also alleviates OxLDL-induced cytotoxicity (28). Jeong *et al.* (29) reported that green tea was also effective in suppressing the apoptosis of neuroblastoma cells, SH-SY5Y cells.

Anti-apoptotic effect After the sample was treated with 4-HNE 50 μM, the cell death also occurred at an initial time of culture and the morphological changes featuring the necrosis were severe after 48 hr. But this was alleviated using GTE 20 μg/mL. Also in the experiment where the

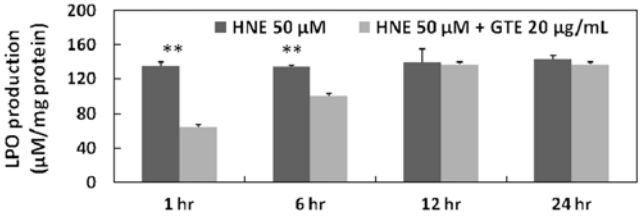

Fig. 3. Protective effects of GTE (20 μg/mL) on the 4-HNE-induced LPO production (μM/mg protein) in ECV 304 cells. GTE treatment significantly (**$p<0.01$) reduced LPO production compared to 4-HNE until 6 hr incubation time.

presence of apoptosis was confirmed with H33342 immunofluorescence study, the progression of apoptosis was demonstrated using DHA (data not shown). In pilot study, the results of caspase-3 activity which was performed to identify the basic mechanisms by which LPO causes apoptosis were as follows: when the sample was treated with 40 μM LPO for 1, 8, 12, 24, and 48 hr, the activity of caspase-3 was increased by 2.3 times (19,764 RFLU) from 8 hr on compared to the control group (8,651 RFLU). The activity of caspase-3 was increased by 3 times (26,010 RFLU) compared to the control group. While the activity of caspase-3 was persisted for 24 hr, it was decreased to 8 hr level at a 48 hr treatment. But these measurements were converted to the percentile value relative to the control group, as shown in Fig. 4A. To put this in another way, the activity of caspase-3 was increased following the treatment with LA and DHA 50 μM/L compared to that 10 μM/L LPO except 48 hr. This was assumed to be a series of apoptosis associated with the damage of mitochondria as an initial mechanism which responds to the oxidative stress mitochondria. The expression of MARK, an early stage of signal transduction system, was examined. This showed that no significant changes in the expressions of p38 MAP kinase and extracellular signal-regulated kinase (ERK), except for that of C-jun N-terminal kinase (JNK), were observed in association with LPO treatment (Fig. 4B). Based on these findings, it can therefore be inferred that LPO is involved in the mechanisms of apoptosis by increasing the activity of JNK, an early stage of signal transduction system.

The activity of caspase, which was formerly increased in the group where 4-HNE was treated, was reduced by the treatment with GTE. Based on the findings that the amount of phophorylated I-kappa B (IκB) was increased, the

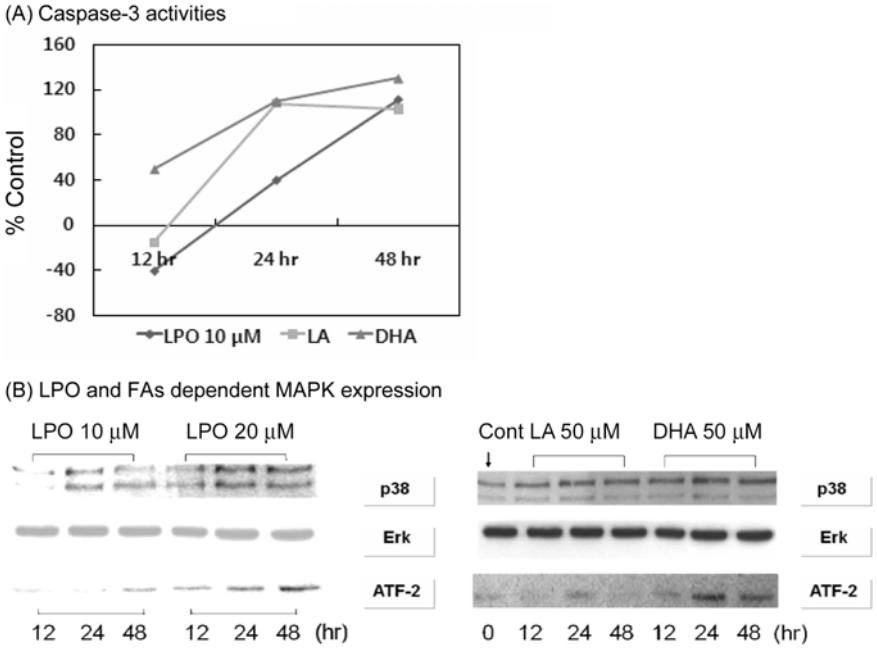

Fig. 4. Caspase-3 activities (% control) in 10 μM LPO and 50 μM of FAs treated groups (A) and LPO concentration-dependent selective activation of MAPK compared to FAs treatment (B) in ECV 304 cells. JNK activity was activated by higher concentration of LPO and DHA as indicated but p38 and ERK kinase were not activated.

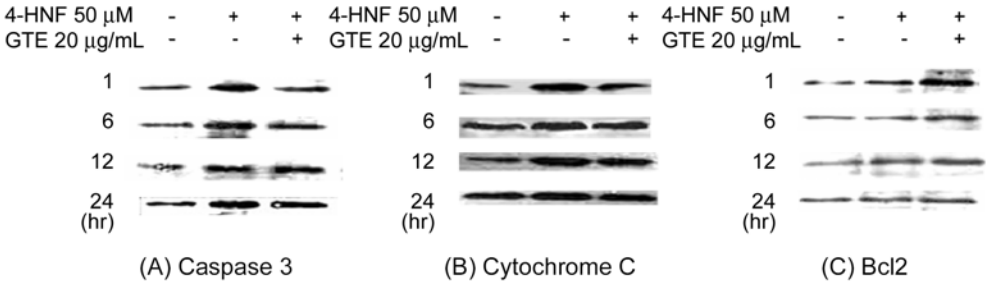

Fig. 5. Effects of GTE 20 μg/mL on the expression of mitochondria damaged apoptotic proteins according to increasing incubation times in 4-HNE induced oxidative stress of ECV 304 cells.

activity of nuclear factor kappa B (NFκB) was confirmed to increase. This is associated with caspase-related signal transduction system which was affected by the production of cytochrome C by mitochondria (data not shown). That is, these findings are identical to the progression to stress mechanism that activate the expression of JNK with the mediation of caspase activity stimulated by 4-HNE. NFκB is a major factor that further progresses the atherosclerotic changes in association with the oxidation-reduction reactions, and it activates the transcription factors for mitogen-activated proteins, MAPK and AP-1. It can therefore be inferred that HNE-induced platelet-derived growth factor (PDGF) synthesis is regulated by NFκB. This reflects the decreased effect by GTE. Moreover, the expression of mitochondrial damage signaling such as cytochrome C related caspase-3 releasing was depressed by GTE and anti-apoptotic protein, Bcl2, was increased until 1-6 hr to a significant extent even though it showed no significant difference compared to controls after (Fig. 5A, B, and C).

As the effects associated with apoptotic proteins, 4-HNE caused a series of apoptosis of caspase-3/-8/-9 in association with mitochondrial damage such as cytochrome C releasing. But green tea suppressed this, as reported in other studies (30,31). Particularly, Lie *et al.* (31) maintained that the activity of caspase-3 due to the release of cytochrome C in mitochondria was associated with the decreased concentration of glutathione peroxidase. Jeong *et al.* (28) also reported that the activity of caspase-3 was increased by OxLDL whose level was elevated by the concurrent presence of inflammatory responses and it was suppressed by various flavonoides including green tea. Cytochrome C is definitely increased during an early stage of culture period as the concentration of LPO, one of the oxidative stress-related factors, is increased. Its concentration was decreased by green tea. Besides, the expression of Bcl2, anti-apoptotic factor based on green tea, blocks the activation of cytochrome C consituting the lower level of mechanism and thereby suppresses the apoptosis. The expression of Bcl2 was increased in the groups where EGCG, quercertin, and hesperetin were concomitantly treated of various flavonoides (28). Our results that the increased expression of Bcl2 blocked cytochrome C - caspase-3/-8/-9 cascade were observed at a 1 and 6 hr culture. No further suppression of the expression of apoptotic protein might be associated

with immediate early genes (IEG) (32). IEGs such as c-Jun or c-Fos are key biomarkers for apoptosis that are closely related to the growth, differentiation and development of cells. IEGs, whose expression was increased, because of oxidative stress increased the phosphorylation of JNK and elevated the amount of its substrate, activating transcription factor-2 and thereby promoted a series of NFκB - cytochrome C - caspase-3/-8/-9 cascade. All of these phases were noted to be blocked by green tea.

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References

1. Seifried HE, Anderson DE, Fisher EI, Milner JA. A review of the interaction among dietary antioxidants and reactive oxygen species. J. Nutr. Biochem. 18: 567-579 (2007)
2. Clement B. Living Foods for Optimum Health: A Highly Effective Program to Remove Toxins and Restore Your Body to Vibrant Health. Prima Press, Rocklin, CA, USA. pp.1-288 (1996)
3. Yam TS, Shah S, Hamilton-Miller JM. Microbiological activity of whole and fractionated crude extracts of tea (*Camellia sinensis*), and of tea components. FEMS. Microbiol. Lett. 152: 169-174 (1997)
4. Ingram D, Sanders K, Kolybaba M, Lopez D. Case-control study of phyto-oestrogens and breast cancer. Lancet 350: 990-994 (1997)
5. Gábor M. Pharmacologic effects of flavonoids on blood vessels. Angiologica 9: 355-374 (1972)
6. Hausteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem. Pharmacol. 32: 1141-1148 (1983)
7. Lavy A, Fuhrman B, Markel A, Dankner G, Ben-Amotz A, Presser D, Aviram M. Effect of dietary supplementation of red or white wine on human blood chemistry, hematology, and coagulation: Favorable effect of red wine on plasma high-density lipoprotein. Ann. Nutr. Metab. 38: 287-294 (1994)
8. Demrow HS, Slane PR, Folts JD. Administration of wine and grape juice inhibits *in vivo* platelet activity and thrombosis in stenosed canine coronary arteries. Circulation 91: 1182-1188 (1995)
9. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen elderly study. Lancet 342: 1007-1011 (1993)
10. Kuriyama S, Shimazu T, Ohmori K, Kikuchi N, Nakaya N, Nishino Y, Tsubono Y, Tsuji I. Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: The Ohsaki study. J. Am. Med. Assoc. 296: 1255-1265 (2006)
11. Imai K, Nakachi K. Cross sectional study of effects of drinking green tea on cardiovascular and liver diseases. Brit. Med. J. 310: 693-696 (1995)
12. Murray M, Pizzorno J. Encyclopedia of Natural Medicine. 2nd ed. Prima Press, Rocklin, CA, USA. pp.1-71 (1998)
13. Wang ZY, Huang MT, Lou YR, Xie JG, Reuhl KR, Newmark HL, Ho CT, Yang CS, Conney AH. Inhibitory effects of black tea, green tea, decaffeinated black tea, and decaffeinated green tea on ultraviolet B light-induced skin carcinogenesis in 7,12-dimethylbenz[a]anthracene-initiated SKH-1 mice. Cancer Res. 54: 3428-3435 (1994)
14. Schneider C, Tallman KA, Porter NA, Brash AR. Two distinct pathways of formation of 4-hydroxynonenal. Mechanisms of nonenzymatic transformation of the 9- and 13-hydroperoxides of linoleic acid to 4-hydroxyalkenals. J. Biol. Chem. 276: 20831-20838 (2001)
15. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde, and related aldehydes. Free Radical Bio. Med. 11: 81-128 (1991)
16. Ruef J, Moser M, Bode C, Kübler W, Runge MS. 4-Hydroxynonenal induces apoptosis, NF-kappaB-activation, and formation of 8-isoprostane in vascular smooth muscle cells. Basic Res. Cardiol. 96: 143-150 (2001)
17. Han CY, Park SY, Pak YK. Role of endocytosis in the transactivation of nuclear factor-kappaB by oxidized low-density lipoprotein. Biochem. J. 350: 829-837 (2000)
18. Luckey SW, Taylor M, Sampey BP, Scheinman RI, Petersen DR. 4-Hydroxynonenal decreases interleukin-6 expression and protein production in primary rat kupffer cells by inhibiting nuclear factor-kappaB activation. J. Pharmacol. Exp. Ther. 302: 296-303 (2002)
19. Vacaresse N, Vieira O, Robbesyn F, Jürgens G, Salvayre R, Negre-Salvayre A. Phenolic antioxidants Trolox and caffeic acid modulate the oxidized LDL-induced EGF-receptor activation. Brit. J. Pharmacol. 132: 1777-1788 (2001)
20. Kim YS, Han CY, Kim SW, Kim JH, Lee SK, Jung DJ, Park SY, Kang H, Choi HS, Lee JW, Pak YK. The orphan nuclear receptor small heterodimer partner as a novel coregulator of nuclear factor-kappa b in oxidized low density lipoprotein-treated macrophage cell line RAW 264.7. J. Biol. Chem. 276: 33736-33740 (2001)
21. Lee M, Bae MA. Docosahexaenoic acid induces apoptosis in CYP2E1-containing HepG2 cells by activating the c-Jun N-terminal protein kinase related mitochondrial damage. J. Nutr. Biochem. 18: 348-354 (2007)
22. Sung M, Kim I, Park M, Whang Y, Lee M. Differential effects of dietary fatty acids on the regulation of CYP2E1 and protein kinase C in human hepatoma HepG2 cells. J. Med. Food. 7: 197-203 (2004)
23. Rah DK, Han DW, Baek HS, Hyon SH, Park JC. Prevention of reactive oxygen species-induced oxidative stress in human microvascular endothelial cells by green tea polyphenol. Toxicol. Lett. 155: 269-275 (2005)
24. Nakashima I, Liu W, Akhand AA, Takeda K, Kawamoto Y, Kato M, Suzuki H. 4-Hydroxynonenal triggers multistep signal transduction cascades for suppression of cellular functions. Mol. Aspects Med. 24: 231-238 (2003)
25. Anuradha CD, Kanno S, Hirano S. Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. Free Radical Bio. Med. 31: 367-373 (2001)
26. Kaneko T, Matsuo M, Baba N. Inhibition of linoleic acid hydroperoxide-induced toxicity in cultured human umbilical vein endothelial cells by catechins. Chem. Biol. Interact. 114: 109-119 (1998)
27. Grinberg LN, Newmark H, Kitrossky N, Rahamim E, Chevion M, Rachmilewitz EA. Protective effects of tea polyphenols against oxidative damage to red blood cells. Biochem. Pharmacol. 54: 973-978 (1997)
28. Jeong YJ, Choi YJ, Kwon HM, Kang SW, Park HS, Lee M, Kang YH. Differential inhibition of oxidized LDL-induced apoptosis in human endothelial cells treated with different flavonoids. Brit. J. Nutr. 93: 581-591 (2005)
29. Jeong JH, Kim HJ, Lee TJ, Kim MK, Park ES, Choi BS. Epigallocatechin 3-gallate attenuates neuronal damage induced by 3-hydroxykynurenine. Toxicology 195: 53-60 (2004)
30. Ji C, Amarnath V, Pietenpol JA, Marnett LJ. 4-Hydroxynonenal induces apoptosis via caspase-3 activation and cytochrome c release. Chem. Res. Toxicol. 14: 1090-1096 (2001)
31. Liu W, Kato M, Akhand AA, Hayakawa A, Suzuki H, Miyata T, Kurokawa K, Hotta Y, Ishikawa N, Nakashima I. 4-Hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. J. Cell Sci. 113: 635-641 (2000)
32. Dragunow M, Young D, Hughes P, MacGibbon G, Lawlor P, Singleton K, Sirimanne E, Beilharz E, Gluckman P. Is c-Jun involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? Brain Res. Mol. Brain Res. 18: 347-352 (1993)