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Methyl gallate and chemicals structurally related to methyl gallate protect human umbilical vein endothelial cells from oxidative stress

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Abbreviations: CAT, catalase; DCFH-DA, 2',7'-dichlorodihydrofluorescein-diacetate; DDRT-PCR, differential display reverse transcription-PCR; EGCG, (-)-epigallocatechin gallate; GPX, glutathione peroxidase; HUVECs, Human umbilical vein endothelial cells; LDL, low density lipid; LPO, lipid peroxidation; MDA, malondialdehyde; meGAL, methyl gallate; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase

Abstract

Methyl gallate (meGAL) is known as one of major antioxidants. To investigate whether meGAL protects human cells from oxidative stress, meGAL extracted from Korean medicinal plant, *Cercis chinensis* leaves, was primarily screened using cell viability assay against oxidative stress. Human umbilical vein endothelial cells (HUVECs) were treated with three different concentrations of meGAL for indicated time. After or during meGAL treatment, H_2O_2 was added and incubated. meGAL showed free radical scavenging effect at low concentration (0.02 mM) and cell protective effect against H_2O_2-mediated oxidative stress. meGAL recovered viability of HUVECs damaged by H_2O_2-treatment, reduced the lipid peroxidation (LPO) and decreased the internal reactive oxygen species (ROS) level elevated by H_2O_2-treatment. Free radical scavenging effect of meGAL was proven to be very high. Differential display reverse transcription-PCR analysis showed that meGAL upregulated the levels of regulator of chromatin condensation 1, type 1 sigma receptor and phosphate carrier protein expressions, respectively. Based on structural similarity compared with meGAL, 14 chemicals were chosen and viability assay was performed. Four chemicals, haematommic acid (56.2% enhancement of viability), gallic acid (35.0%), methylorsellinic acid (23.7%), and syringic acid (20.8%), enhanced more potent cell viability than meGAL, which showed only 18.1% enhancement of cell viability. These results suggest that meGAL and four meGAL-related chemicals protect HUVECs from oxidative stress.

Keywords: gallic acid; hydrogen peroxide; methyl gallate; oxidative stress; plant, medicinal; syringic acid

Introduction

ROS and reactive nitrogen species (RNS) constantly generated in normal condition by aerobic metabolism include free radicals such as superoxide anion, hydroxyl radicals, nonradical hydrogen peroxide, peroxinitrite, nitroxyl anion and nitric oxide (Beckman and Ames, 1998; Curtin *et al.,* 2002; Droge, 2002). In normal biological systems, redox homeostasis is maintained by controlling the balance between ROS production and various types of scavengers called antioxidants. Transient changes in oxidants-antioxidant balance are normally regulated by changing the production of counter species and reached to the steady-state over time (Shuli *et al.,* 1991; Nakamura *et al.,* 1994; Adler *et al.,* 1999; Zhang and Storz, 2000; Droge, 2002). The persistent production of abnormally large amount of ROS or RNS, however,

may lead to persistent changes in signal transduction and gene expression, which in turn may give rise to certain diseases.

meGAL is known as one of major antioxidants (Westenburg *et al.,* 2000; Galato *et al.,* 2001; Cho *et al.,* 2003; Sohi *et al.,* 2003). meGAL is a phytochemical from various species including *Meliaceae, Rosa Rugosa* and *Galla Rhois*, and its derivative (-)-epigallocatechin gallate (EGCG) is a major pytochemical of green tea, well known antioxidative beverage (Chen and Zhang, 2003). The radical scavenging effect and inhibition of LPO have been reported as major activities of meGAL (Westenburg *et al.,* 2000; Galato *et al.,* 2001; Cho *et al.,* 2003). In addition, meGAL possesses the biological activities including antiplatelet activity (Lim *et al.,* 2004), protection of DNA damaging against oxidative stress (Hsieh *et al.,* 2004), protection of lung injury induced by phosgene (Sciuto and Moran, 2001), attenuation of diabetic oxidative stress (Cho *et al.,* 2004) and antiapoptotic activity (Sohi *et al.,* 2003). However, radical scavenging ability of meGAL has been a main focus to explain its protective function against oxidative stress.

The endothelium is known to be sensitive to injury caused by ROS (Valen et al., 1999), and in contrast, free radicals released by endothelial cells mediate the oxidation of low density lipid (LDL) (Zapolska-Downar *et al.,* 1999). HUVECs have been used to study oxidative stress related researches and oxidative stress mediated by H_2O_2 damages cellular functions of HUVECs via various mechanisms (Valen *et al.,* 1999; Estrada-Garcia *et al.,* 2002; Waxman *et al.,* 2003).

Various enzymatic and non-enzymatic self defense systems against oxidative stress including transiently over-produced ROS inside cells or exposure to external ROS inducing species have been identified and studied extensively. Catalase (CAT), SOD, and glutathione peroxidase (GPX) are the examples of enzymatic defense systems and non-enzymatic systems include tocopherol, ascorbate, urate and glutathione (Sies, 1993).

In the present study, we investigated cellular protective activity of meGAL under oxidative stress, identified genes expressed differentially by meGAL treatment and compared antioxidant effect of meGAL with its structurally related chemicals.

Materials and Methods

Chemicals

meGAL was extracted from *Cercis chinensis* leaves, purified and characterized as described (Kizu and Tomimori, 2003). meGAL (gallicin) was isolated from 2 kg of *Cercis chinensis* leaves. Dried leaves of the plants were extracted with methanol. The methanol extract was suspended with water and subsequently partitioned with ether. With 45 g of ether layer, Sephadex LH-20 column chromatography was repeatedly operated and finally 4.2 g of pure compound was obtained. Ethanol was used as a developing solvent. The structure of compound was elucidated by spectroscopic parameters of ^{1}H-NMR, ^{13}C-NMR, FT-IR and FAB-MS, and identified as meGAL.

HUVEC culture

Cells were isolated from umbilical cords during full-term delivery. Patient consent was obtained from each individual and the use of tissue samples was approved by the ethics committee of our institution. Veins were cannulated and flushed first with PBS (170 mM NaCl, 10 mM Na_2HPO_4, 3.3 mM KCl, and 1.8 mM KH_2PO_4, pH 7.4) before being filled with 0.2% (v/v) collagenase type II (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS). Following 10 min incubation at 37°C (5% CO_2), the collagenase was removed by flushing with M-199 medium, and cells were centrifuged for 10 min at 1,000 g. Cells were then resuspended in growth medium (M-199) and seeded into 25-cm^2 cell cultured flask previously coated with 1% (v/v) liquid gelatin (Sigma) with PBS. Cells were grown to confluence before starting the treatments.

Cell viability assay

HUVECs were grown in 96-well micro plates in a final volume of 100 μl culture medium containing 10^4 cells per well. After allowing the cultures to grow to confluence, chemical and H_2O_2 treatments were followed with certain intervals. To determine the cell viability, XTT assay kit was used as described in company's manual (R&D Systems Inc., Minneapolis, MN). Two cases of oxidative stress were applied to pre-incubated HUVECs with chemical for 1 h: the treatment with 1 mM H_2O_2 for 1 h and the treatment with 0.1 mM H_2O_2 for 48 h. Concentrations of chemical were varied from 0 to 0.5 mM.

CAT activity

CAT activity was determined using Oxis Research kit (OXISResearch, Portland, OR). After addition of 10 mM H_2O_2, cell lysates were incubated for 1 min and mixed with chromogen substrate followed by addition of stopping reagent. Developed color for 10 min incubation was detected at 520 nm. The rate of change in absorbance was converted to units of enzyme activity, determined from a standard curve using CAT. Enzyme activity was then standardized to mg protein.

SOD enzymatic activity

SOD enzymatic activity was determined using the assay described in Oxis research SOD-525 kit manual (OXISResearch, Portland, OR). In brief, cell lysates were treated with mercaptan eliminating reagent and chromogenic substrate of SOD was added to the samples. SOD activity was determined by measuring the absorbance at 525 nm over time.

Lipid peroxidation assay

The extent of lipid peroxidation was determined by using the assay described in Oxis research LPO-586 kit manual (OXISResearch, Portland, OR). In brief, the LPO-586 assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenyliindole with malondialdehyde (MDA) and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-hydroxyalenal reacts with 2 molecules of reagent, N-methyl-2-phenyliindole, to yield a stable chromophore with maximal absorbance at 586 nm.

Determination of intracellular ROS

Intracellular ROS levels were determined by ROS mediated conversion of non-fluorescent 2',7'-dichlorofluorescein diacetate (DCFH) to DCF. The study was performed by slight modification of a method described previously (Zapolska-Downar *et al.*, 1999). Cells were cultured overnight in 6-well culture plates and preincubated with meGAL for 1 h. Next, 1 mM of H_2O_2 was added to the cells and the cells were incubated for 1 h. The cells were washed with M-199 media twice and followed by incubation with 0.02 mM DCFH for 30 min in the dark. After incubation, 3 times of washing and lysis of the cells with 0.1% Triton X-100 in 0.1 M Tris was carried out. Relative fluorescence intensity of DCF at an emission of 525 nm and an excitation of 475 nm of each sample was measured using Victor V^3 (Perkin-Elmer).

Free radical scavenging capacity

1,1-diphenyl-3-picrylhydrazyl (DPPH) radical has a deep violet color due to its unpaired electron and radical scavenging can be followed spectrophotometrically by the loss of absorbance at 517 nm as the pale yellow non-radical form is produced. 0.08 mM of DPPH solution in ethanol was prepared and added to various concentrations of chemicals in ethanol. Absorbance changes at 517 nm for 10 min with 10 s of interval were monitored.

Differential display reverse transcription-polymerase chain reaction (DDRT-PCR)

For DD of mRNA (Liang and Pardee, 1992), HUVECs were obtained from full-term placenta after delivery. Patient consent was obtained from each individual and the use of tissue samples was approved by the ethics committee of our institution. Total RNA was extracted from HUVECs treated with or without 0.2 mM of meGAL for 8 h using an RNA extraction kit (RNeasy total RNA kit; Qiagen Inc., Valencia, CA) and 0.2 μg of total RNA was used to generate cDNA in a reverse transcription reaction (RNAimage^{TM} kit, GenHunter, MA). With the use of the differential display kit (RNAimage^{TM} kit), we performed PCR using oligo-dT primers and arbitrary sequences, each 13 bases in length according to the manufacturer's recommendations. After cDNAs of 3′ termini of mRNAs were generated, the PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel. From the films, differentially expressed genes, which were overexpressed or down-regulated in various chemicals and H_2O_2 treated HUVECs, were identified and were then subcloned into the pGEM-T easy vector with the use of the TA cloning system, and subjected to an automatic sequencing analysis.

Northern blot analysis

The extracted total RNA of the cells was treated with DNase I and reverse transcribed with random hexamer priming (Clontech). By using the product as template, PCR was performed by using 5′-GTGAG-CGCAAAGCCTCAG-3′ as the sense primer and 5′-TGCATGGTGTATGTCCCTGT-3′ as the antisense primer (Sig-1R GenBank accession no. NM_005866).This RT-PCR product size was 840 bp. This 840-bp product was used as a probe for Northern blot analysis. Northern blot analysis was carried out, in which 20 μg of denatured total RNA was electrophoresed on a 1.0% formaldehyde agarose gel and transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). Blot was hybridized with the randomly primed [^{32}P]-labeled 840-bp cDNA probe. Human β-actin cDNA control probe was used as a loading control.

Cell viability screening with chemical library

Based on structural similarity compared with meGAL, 14 chemicals showing more than 70% structural similarity were chosen. These 14 chemicals were kindly provided by Dr. HS Park (Dept. of Chemistry, Yale University). Structural similarity was calculated by ChemFinder (Cambridgesoft, MA). Basically same experimental procedure with previous cell viability assay was used for meGAL-related chemical screening except that the concentration of each chemical and H_2O_2 was fixed at 0.02 mM and 0.2 mM, respectively. Chemicals were pretreated to HUVECs

for 1h and then cells were incubated with 0.2 mM H_2O_2 for 1 h. Less harsh condition (0.2 mM) than 1 mM of H_2O_2 was used to increase selection ratio in this primary screening, which would give more information about the relationship between chemical structures and activities. HUVECs' survival was determined as about 60% at 0.2 mM H_2O_2 treatment. Experiments for each chemical were repeated three times and data were averaged. Viability recovery was calculated as relative recovery ratio, which means differences from cell viability of control. Control is viabilities of cells treated with H_2O_2 alone.

Results

Effects of H_2O_2 exposure on HUVECs in cell based assay

Cell based assay is basically to monitor the change of cell viability induced by oxidative stress, which was H_2O_2 exposure on HUVECs in our experiments.

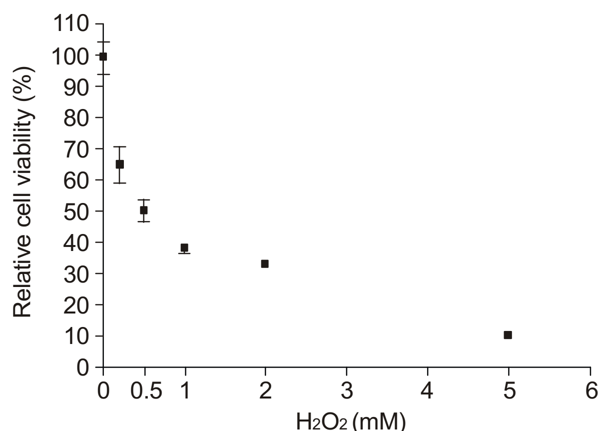

Figure 1. Determination of relative cell viability after treatment with H_2O_2. Various concentrations of hydrogen peroxide (0-5 mM) were treated into HUVEC culture media and cell viability was assessed. 0.5 mM of H_2O_2 induced about 50% cell death and 1 mM of H_2O_2 resulted in about 60% cell death. Each value is the means ± SD of three independent experiments.

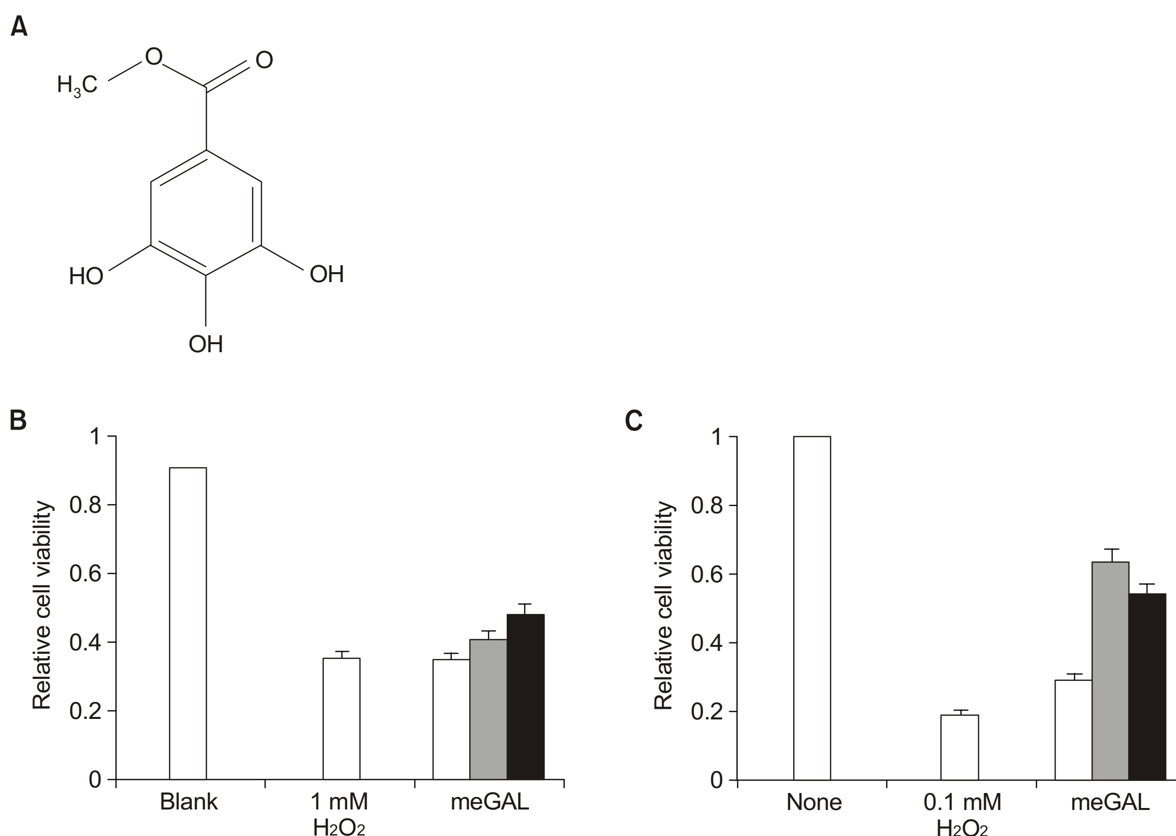

Figure 2. Primary screening of meGAL using cell based assay. (A) The chemical structure of meGAL. Cell viability assay was performed with three different concentrations of meGAL. Prior to incubation with H_2O_2, HUVECs were treated with 0.02 (opened bar), 0.1 (scratched bar) and 0.5 (black bar) mM of each phytochemical for 1 h and then followed (B) 1 h incubation in 1 mM H_2O_2 and (C) 48 h incubation in 0.1 mM H_2O_2. Blank denotes untreated control HUVECs. Data are represented as mean ± SD.

Preliminary experiments were performed to determine the effects of H_2O_2 exposure on HUVECs (Figure 1). The concentration of H_2O_2 was varied from 0.2 to 5 mM and cell death induced by H_2O_2 was monitored. Cell viability was also assessed after 0.5, 1 or 2 h. Cell viability was ranged from 70% (0.2 mM) to 10% (5 mM) and 40% of cells were viable at 1 mM H_2O_2 (Figure 1). Therefore, 1 mM H_2O_2 was used through all subsequent screenings to differentiate the effects of survival and death by meGAL.

Primary screening of meGAL using cell based assay

meGAL was extracted, purified and used as a primary screening material (Figure 2A). Since cell based assay is more direct assay to select antioxidative chemicals, it was employed as primary screening assay. Three different concentrations (0.02, 0.1, and 0.5 mM) of meGAL were treated for 1 h prior to the addition of 1 mM H_2O_2 and relative cell viabilities in comparison to HUVECs alone were monitored (Figure 2B). In addition to high concentration of H_2O_2 treatment (1 mM), low concentration of H_2O_2 (0.1 mM) was introduced on HUVECs for 48 h to evaluate meGAL that may protect cells exposed to H_2O_2 at low concentration and for 48 h (Figure 2C). meGAL improved cell survival rate at 0.1 mM and 0.5 mM concentrations, respectively (Figures 2B and 2C). Especially, for long exposure and at low concentration of H_2O_2 (0.1 mM, 48 h), meGAL enhanced the viability of HUVECs up to about 60% (Figure 2C) when compared with the survival rate of HUVECs exposed to 1 mM H_2O_2 for 1 h (40%) or exposed to 0.1 mM H_2O_2 for 48 h (20%).

SOD and CAT assays

In the present experiment, H_2O_2 exposure on HUVECs resulted in no significant change in either CAT or SOD activities in HUVEcs and meGAL also did not affect activities of both enzymes in HUVECs (data not shown).

LPO assay and intracellular H_2O_2 determination

MDA level obtained from meGAL treatment on HUVECs alone was similar to the level in the HUVEC cultures without oxidative stress (Figure 3A). When the cells are treated with H_2O_2, however, there was a significant increase of MDA production to about 210% (P = 0.0317). This elevated MDA level was decreased to180% with treatment of meGAL prior to the addition of H_2O_2 (Figure 3A).

To address the possibility that the increased cell viability in the presence of phytochemicals is due to decreased production of ROS inside cells, we measured intracellular concentrations of H_2O_2 in HUVECs. The effect of meGAL on cellular oxidation was determined by DCF fluorescence. HUVECs activated by H_2O_2 showed an increase in free radical level by about 55% over non-treated HUVECs (Figure 3B). Pretreatment with meGAL prior to the addition of H_2O_2 decreased intracellular H_2O_2 levels to 24% (Figure 3B). Since meGAL showed high ROS scavenging effect, direct scavenging of ROS by meGAL could account for reduced intracellular H_2O_2.

Free radical scavenging activity

meGAL exposure on HUVECs showed very high free radical scavenging activity at low concentrations

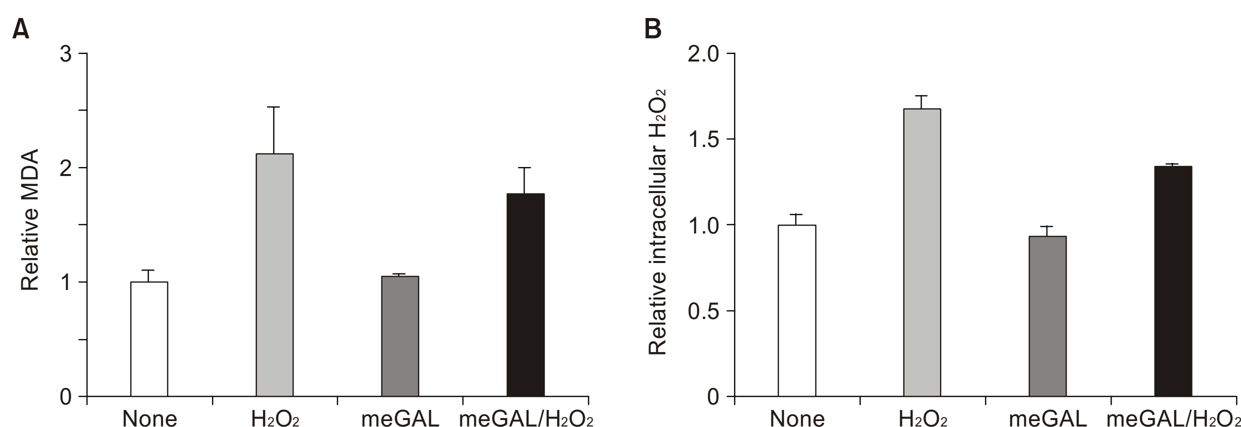

Figure 3. Intracellular LPO production and H_2O_2 determination. (A) LPO production of HUVECs with the treatment with H_2O_2 and/or meGAL. P = 0.0317 by one-sided t-test with normalized values. (B) Intracellular H_2O_2 determination by DCF with the treatment with H_2O_2 and/or meGAL for 1 h. Each value is the means ± SD of three independent experiments.

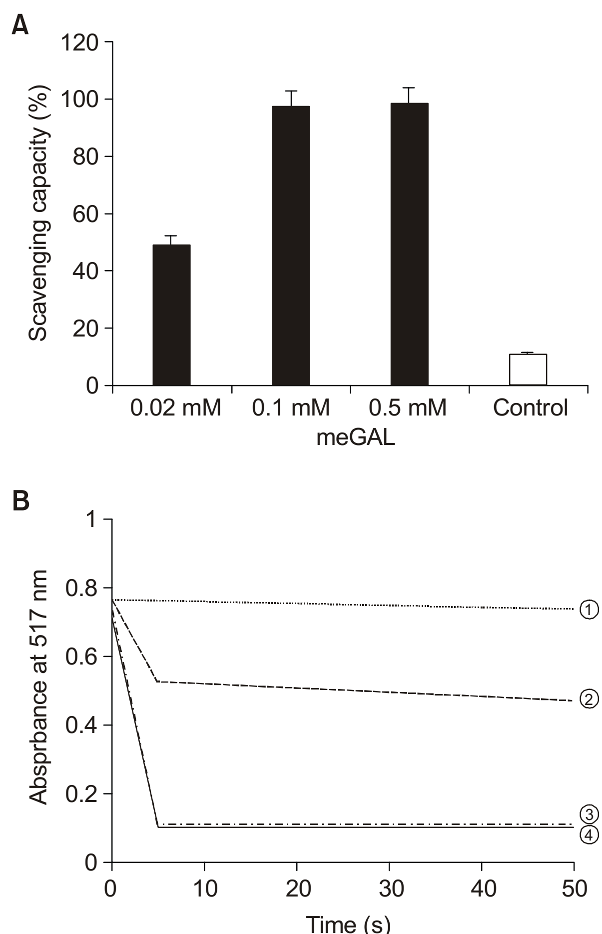

Figure 4. Free radical scavenging activity and scavenging kinetics of meGAL. (A) Free radical scavenging ability of meGAL determined by using DPPH assay. 80 μM of DPPH solution in ethanol was prepared and added to various concentrations of chemical (0.02, 0.1 and 0.5 mM) in ethanol. Absorbance changes at 517 nm for 10 min with 10 s of interval were monitored. Each value is the means ± SD of three independent experiments. (B) Time dependent free radical scavenging by meGAL. After mixing DPPH and meGAL, with 5 s intervals absorbance at 517 nm was monitored. DPPH alone (①), with 0.02 mM (②), 0.1 mM (③), 0.5 mM of meGAL (④).

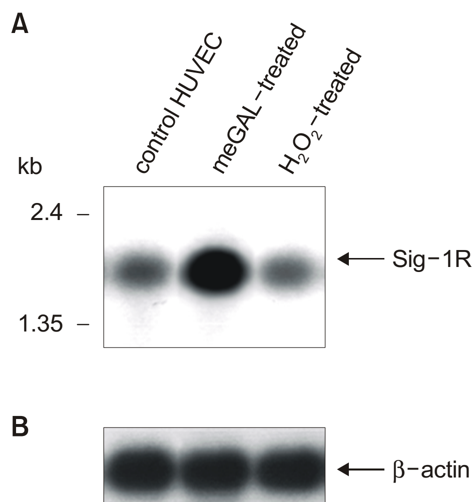

Figure 5. Sig-1R gene expressions in HUVECs. Northern blot analysis was performed to determine the expression pattern in HUVECs. Blot was hybridized with the randomly primed [^{32}P]-labeled 840-bp cDNA probe. Human β-actin cDNA control probe was used as a loading control.

Table 1. Patterns of differentially expressed genes by meGAL or H_2O_2.

Expression			Gene
Control	meGAL	H_2O_2	
Weak	Strong	Weak	Type I Sigma receptor (Sig-1R)
Weak	Medium	Weak	Chromosome condensation 1 (RCC1)
Weak	Medium	Weak	Phosphate carrier

(Figure 4A). At 0.02 mM of meGAL, 50% of free radicals were quenched and over 0.1 mM, free radical scavenging was saturated. In terms of scavenging kinetics, free radical scavenging by meGAL was immediate. Within 5 s, more than 90% of free radicals were scavenged at 0.02 mM of meGAL exposure on HUVECs (Figure 4B). Consequently, free radical scavenging ability of meGAL was fast and efficient at used concentrations, which may contribute on the cell protective ability of meGAL against oxidative stress.

Differential display and Northern blot analysis

Using the DDRT-PCR, genomic expression levels in HUVECs were estimated after H_2O_2 exposure or meGAL treatment, respectively. Differentially expressed mRNAs by meGAL or H_2O_2 treatment were selected and compared to each other. Among identified genes, the change of gene expression pattern by meGAL in comparison to untreated HUVECs was interesting and unique. meGAL upregulated various membrane receptor gene expressions including regulator of chromatin condensation 1 (RCC1), type 1 sigma receptor (Sig-1R) and phosphate carrier protein (PiC) (Table 1). Among three identified genes, RCC1, Sig-1R and PiC, by using the DDRT-PCR, Sig-1R showed the most strong upregulation after treatment with meGAL compared with RCC1 or PiC (Table 1). Accordingly we performed Northern blot analysis to reconfirm the upregulation of Sig-1R in meGAL-treated HUVECs. As expected, Sig-1R was upregulatedd in meGAL-treated HUVECs compared to control HUVECs or H_2O_2-treated HUVECs (Figure 5).

Table 2. Screening results of meGAL-related chemical library.

No. of chemical	Structure	Name	Survival[a] (%)	No. of chemical	Structure	Name	Survival[a] (%)
1		EVERNINIC ACID	12.6	9		GENETISIC ACID	15.9
2		METHYLORSE LLINIC ACID, ETHYL ESTER	23.7	10		SODIUM *p*-AMINOSALIC YLATE	16.8
3		GALLIC ACID	35.0	11		ASPIRIN	5.9
4		HAEMATOMMIC ACID, ETHYL ESTER	56.2	12		BENZOCAINE	11.7
5		SYRINGIC ACID	20.8	13		BENZYL BENZOATE	7.5
6		OBLIQUIN	10.5	14		DIPYROCETYL	5.9
7		ATRANORIN	13.0	meGAL		Methyl gallate	18.1
8		FRAXETIN	11.7				

[a]Survival: relative survival ratio (%) to control.

Cell viability assay with chemicals structurally related to meGAL

Based on structural similarity compared with meGAL, 14 chemicals showing more than 70% structural similarity were chosen and previous cell viability assay was used for meGAL-related chemical screening (Table 2). To determine the cell viability, XTT assay was used as described. Through viability assay, four chemicals related to meGAL showed more than 20% enhancement of viability in comparison with control (Table 2). Four chemicals related to meGAL were methylorsellinic acid ethyl ester (chemical No. 2, 23.7% enhancement of viability), gallic acid (chemical No. 3, 35.0% enhancement of viability), haematommic acid ethyl ester (chemical No. 4, 56.2% enhancement of viability) and syringic acid (chemical No. 5, 20.8% enhancement of viability). Percentages in parenthesis indicate the relative viability enhancement. Experiments for each chemical were repeated three times and data were averaged. Haematommic acid ethyl ester showed the highest activity among tested 14 chemicals. These 4 chemicals were selected as primary hits since those were considered as chemicals showing significant enhancement of viability in comparison with meGAL, which showed only 18.1% enhancement of cell viability.

Discussion

Reducing reactive oxygen species in the body is one of main efforts towards protection of various diseases such as aging process, cancer, diabetes and neurodegenerative diseases (Hsieh *et al.,* 2004). Many natural products including flavonoids, cumarins, polyols have been studied for the characterization and the development as antioxidative reagents (Finkel and Holbrook, 2000; Lee *et al.*, 2002).

In our studies, meGAL, a polyphenol derivative, showed protective effects against H_2O_2-mediated oxidative stress. Very efficient radical scavenging ability of meGAL clearly explains the protective effect

against oxidative stress. Especially for the long term exposure (48 h) at low concentration (0.1 mM) of H_2O_2, meGAL was very effective. While meGAL did not affect the biological activities involved in antioxidative mechanisms such as CAT and SOD in the biological systems, induced lipid peroxidation and internal ROS level by H_2O_2 in the HUVECs were significantly reduced by meGAL treatment. From these results, H_2O_2 scavenging effect probably is primary reason for the protective effect of meGAL from H_2O_2-mediated oxidative stress.

To find out more efficient antioxidants from the meGAL derivatives, 14 chemicals showing more than 70% structural similarity with meGAL were chosen and cell viability assay was used for meGAL-related chemical screening. Through viability assay, four chemicals related to meGAL showed more than 20% enhancement of viability in comparison with control. Four chemicals related to meGAL were methylorsellinic acid ethyl ester, gallic acid, haematommic acid ethyl ester and syringic acid. Haematommic acid ethyl ester showed the highest enhancement of cell viability (56.2%) among tested 14 chemicals.

Methylorsellinic acid is the precursor of mycophenolic acid which is the active compound formed following the administration of the pro-drug mycophenolate mofetil (Puel *et al.,* 2005). Mycophenolic acid is an antibiotic useful in research for the selection of animal cells that express the *E. coli* gene coding for xanthine guanine phosphoribosyltransferase and is also used as immunosuppressive agent. Plant phenolic, which form moieties on flavonoid rings, such as gallic acid, is widely consumed. Several beneficial properties have been attributed to this dietary compound, including antioxidant, anti-inflammatory, and anticarcinogenic effects (Galati and O'Brien, 2004). Phenolic compounds, hematommic acid and syringic acid, protect brain against lipid peroxidation (Toledo Marante *et al.,* 2003) and show antioxidant activity (Kalkan Yildirim *et al.,* 2004), respectively. These 4 chemicals were selected as primary hits since those were considered as chemicals showing significant enhancement of viability in comparison with meGAL. Further investigation would be necessary to optimize its activity and drug-likeness for the development as a drug candidate.

To link the evidences of antioxidative ability of meGAL to molecular mechanisms, the analysis of gene expression patterns by differential display method was carried out. Our results suggest alternative mechanisms for the antioxidative effects of meGAL. Upregulated genes by meGAL were RCC1, Sig-1R and PiC. RCC1 is known to be involved in cell cycle, cell proliferation, nuclear transport and the inhibition of the Ran-GEF activity of RCC1 was reported to cause cell death through premature chromatin condensation (Nishijima *et al.,* 2003; Zheng, 2004). Sig-1R also has the variety of functions such as a novel opioid receptor, cellular proliferation and modulations of ion channel, ankyrin, Ca^{2+} and sphingolipid levels (Hayashi and Su, 2001; Aydar *et al.,* 2004; Spruce *et al.,* 2004). Similar to RCC1, small molecule antagonists of Sig-1R inhibit tumor cell survival by activating programmed cell death (Zheng, 2004). The fact that inhibitors of RCC1 and Sig-1R induce cancer cell death through various mechanisms suggests that RCC1 and Sig-1R may play important roles in maintaining cell viability. Especially, Sig-1R involved in calcium flux supports its role in cell survival against oxidative stress. Oxidative stress mediated increase in the cytosolic calcium concentration contributes to the activation of PKC-alpha (Larsson and Cerutti, 1989) and to the transcriptional induction of the AP-1, of which pathway has been known as apoptotic process (Droge, 2002). In a recent report, it has been shown that Sig-1R prevents the rise in intracellular calcium concentration and consequently controls PLC activity. In addition to the calcium regulation, antagonists of Sig-1R inhibit the activity of PKB/Akt in an apparently calcium-independent manner. Thus, PLC activation and PKB/Akt inhibition in response to Sig-1R antagonists appear to constitute biochemically separable signal transduction responses, which are to restrain the proapoptotic signaling pathway and to stimulate a prosurvival signaling cascade (Spruce *et al.,* 2004). Based on these results that Sig-1R regulates apoptosis and survival signaling pathways for the cell survival, cellular protective ability of meGAL against oxidative stress may be linked to the induction of Sig-1R gene by meGAL.

These results suggest that meGAL-induced genes may play roles in cellular protective functions of meGAL. Further investigation with identified genes is intensively undergoing to support this preliminary linkage between anti-oxidative activities and molecular mechanisms involved in meGAL-mediated cell survival. This study suggests that meGAL and meGAL-related chemicals protect HUVECs from oxidative stress.

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