Isolation, identification, and biological evaluation of Nrf2-ARE activator from the leaves of green perilla (*Perilla frutescens* var. *crispa* f. *viridis*)

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Abbreviations: antioxidant response element (ARE), 2',7'-dichlorofluorescein (DCF), 2',3'-dihydroxy-4',6'-dimethoxychalcone (DDC), dihydroethidium (DHE), dimethylsulfoxide (DMSO), electron impact (EI), ethidium (ETH), γ-glutamylcysteine synthetase (γ-GCS), reduced glutathione (GSH), 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA), heme oxygenase-1 (HO-1), high-pressure liquid chromatography (HPLC), 6-hydroxydopamine (6-OHDA), kelch-like ECH-associated protein 1 (Keap1), mitogen-activated protein kinase (MAPK), mass spectrometry (MS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nuclear magnetic resonance (NMR), NAD(P)H: quinone oxidoreductase-1 (NQO1), nuclear factor erythroid 2-related factor 2 (Nrf2), oxygen radical absorbance capacity (ORAC), phosphoinositide 3-kinase (PI3K), reactive oxygen species (ROS), small interfering RNA (siRNA), trifluoroacetic acid (TFA).

Abstract

The nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway is a cellular defense system against oxidative stress. Activation of this pathway increases expression of antioxidant enzymes. Epidemiological studies have demonstrated that the consumption of fruits and vegetables is associated with reduced risk of contracting a variety of human diseases. The aim of this study is to find Nrf2-ARE activators in dietary fruits and vegetables. We first attempted to compare the potency of ARE activation in six fruit and six vegetables extracts. Green perilla (*Perilla frutescens* var. *crispa* f. *viridis*) extract exhibited high ARE activity. We isolated the active fraction from green perilla extract through bioactivity-guided fractionation. Based on nuclear magnetic resonance and mass spectrometric analysis, the active ingredient responsible for the ARE activity was identified as 2′,3′-dihydroxy-4′,6′-dimethoxychalcone (DDC). DDC induced the expression of antioxidant enzymes, such as γ-glutamylcysteine synthetase (γ-GCS), NAD(P)H: quinone oxidoreductase-1 (NQO1), and heme oxygenase-1. DDC inhibited the formation of intracellular reactive oxygen species and the cytotoxicity induced by 6-hydroxydopamine. Inhibition of the p38 mitogen-activated protein kinase pathway abolished ARE activation, the induction of γ-GCS and NQO1, and the cytoprotective effect brought about by DDC. Thus, this study demonstrated that DDC contained in green perilla enhanced cellular resistance to oxidative damage through activation of Nrf2-ARE pathway.

Keywords: chalcone; green perilla; Nrf2-ARE pathway; oxidative stress; 6-hydroxydopamine.

Introduction

Oxidative stress, also referred to as a reactive oxygen species (ROS)-antioxidant imbalance, occurs when the net amount of ROS exceeds the antioxidant capacity. Oxidative stress is thought to play a major role in the pathogenesis of a variety of human diseases, including aging, carcinogenesis, metabolic syndrome, cardiovascular, neurodegenerative and kidney diseases [1], [2], [3], [4], [5], and [6]. Epidemiological evidence indicates that a significant reduction in the risk of ischemic stroke, some forms of cancer, and Alzheimer's disease can be obtained by increasing fruit and vegetable consumption [7], [8] and [9]. Therefore, it is widely believed that dietary fruit and vegetable intake is beneficial in preventing disease onset and slowing disease progression.

Vitamins C and E, polyphenols, and carotenoids are thought to be responsible for most of the antioxidant activity in foods. Various studies have suggested that dietary antioxidants may protect against cardiovascular diseases, neurodegenerative diseases, and some forms of cancer [10], [11], [12], and [13]. The direct antioxidant capacities of food extracts have been comparatively assessed using TEAC (Trolox Equivalent Antioxidant Capacity), FRAP (Ferric Reducing Ability of Plasma), and ORAC (Oxygen Radical Absorbance Capacity) assays [14], [15], and [16]. Although the radical-scavenging activities of phytochemicals from foods are responsible, at least in part, for human health-promoting effects, it is unclear whether the direct antioxidant activities of phytochemicals *in vitro* can completely explain their systemic antioxidant effects *in vivo* [17].

Accumulating evidence suggests that many phytochemicals, such as sulforaphane and curcumin, enhance the expression of antioxidant enzymes and cytoprotective proteins, for

example, NAD(P)H: quinone oxidoreductase-1 (NQO1), superoxide dismutase, glutathione S-transferase, glutathione peroxidase, heme oxygenase-1 (HO-1), γ-glutamylcysteine synthetase (γ-GCS), catalase, and thioredoxin [18]. The enhanced expression of antioxidant enzymes and cytoprotective proteins is mainly controlled by the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway. Under normal physiological conditions, Nrf2 is inactive due to binding with the skeletal actin-binding protein, Kelch-like ECH-associated protein 1 (Keap1). Under conditions of oxidative stress, Nrf2 is no longer sequestered by Keap1 and is subsequently translocated to the nucleus and bound to ARE. This results in the transcriptional activation of a number of phase 2 detoxifying and antioxidant enzymes [19]. Nrf2-ARE pathway functions as a cellular defense system against oxidative stress and has recently received attention as a potential therapeutic target for cancer chemoprevention, and cardiovascular and neurodegenerative diseases [20], [21], and [22]. Nevertheless, compared to ORAC values in the database of the U.S. Department of Agriculture's Agricultural Research Service, limited data exists comparing this indirect antioxidant property among foods, although the potency of induction of NQO1 in selected vegetables has been examined [23].

To discover novel Nrf2-ARE activators, the present study was designed to compare the ARE activity of the extracts of fruits and vegetables that are widely consumed in either Eastern or Western food cultures. Fruits and vegetables tested included the following: peach (*Amygdalus persica*), apple (*Malus pumila* Mill.), strawberry (*Fragaria* L.), cranberry (*Vaccinium oxycoccos*), raspberry (*Rubus idaeus*), satsuma mandarin (*Citrus unshiu* Marc.), green perilla (*Perilla frutescens* var. *crispa* f. *viridis*), tossa jute (*Corchorus olitorius* L.), crown daisy (*Glebionis coronaria* L.), celery (*Apium graveolens* var. *dulce*), parsley (*Petroselium crispum*),

and red perilla (*Perilla frutescens* var. *crispa* f.*purpurea*). We carried out a bioactivity-guided

fractionation to isolate the active constituents from the fruit and vegetable extracts. After

chemical identification, the active compounds were evaluated for their protective activity against

6-hydroxydopamine (6-OHDA)-induced cell toxicity.

Materials and Methods

Materials

  Fruit and vegetable samples were obtained from Ehime Beverage (Matsuyama, Japan), Skylight Biotech (Akita, Japan), and Sanyo Foods (Tokyo, Japan). 6-Hydroxydopamine hydrochloride and *trans*-cinnamaldehyde were purchased from Sigma (St. Louis, MO, USA). *trans*-Cinnamoyl chloride and (*E*)-chalcone were obtained from Wako (Osaka, Japan). 2′-Hydroxychalcone was obtained from TCI (Tokyo, Japan). 2′,4′-Dimethoxychalcone, 2′-hydroxy-4′,6′-dimethoxyacetophenone, 2′-hydroxy-4′-methoxychalcone, 2′-hydroxy-6′-methoxychalcone, and 2′,6′-dimethoxychalcone were purchased from Indofine Chemical (Hillsborough, NJ, USA). SB203580 and LY294002 were obtained from Calbiochem (San Diego, CA, USA). Flavokawain B was purchased from LKT (St. Paul, MN, USA). 2′,3′-Dihydroxy-4′,6′-dimethoxychalcone was synthesized and provided by Pharmaeight (Kyoto, Japan).

Cell culture, transfection, and luciferase reporter analysis

  Rat adrenal pheochromocytoma PC12 cells were maintained in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum and 10% horse serum. Cell cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

  PC12 reporter cells were generated by stable transfection of ARE-luciferase construct. The annealed oligonucleotide of the rat NQO1 ARE (top strand: CTCAGAGATTTCAGTCTAGAGTCACAGTGACTTGGCAAAATCA; bottom strand: CATGGAGTCTCTAAAGTCAGATCTCAGTGTCACTGAACCGTTTTAGTTCGA) was

ligated to the KpnI and HindIII site of the pGL4.27 vector (Promega, Madison, WI, USA). The

cells were transfected with the plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA,

USA) according to the manufacturer's instructions. The culture medium was supplemented with

hygromycin B (300 µg/ml) to select drug-resistant stable transfectants. In the reporter gene

assays, firefly luciferase activity in cell lysates was measured with a luminometer using

Picagene LT 2.0 Luminescence Reagent (Toyo Ink, Tokyo, Japan).

Preparation of fruit and vegetable extracts

The extraction solvent chosen in this study was diethylether, which is useful for

extracting low molecular weight compounds [24]. Raw leaves (100 g) were oven dried at 75°C

and pulverized, and then were suspended in distilled water (200 mL). Ether/water extraction

was performed by adding an equal volume of diethylether to the aqueous mixture from juice

(100 mL) or raw leaves (200 mL) and rigorously shaking for a few minutes using a separating

funnel. The ether layer was evaporated to dryness in a vacuum rotary evaporator. Solvent

extraction was repeated at least thrice. The residues were transferred to a glass tube and

evaporated to dryness under nitrogen gas. The extracts were dissolved in dimethylsulfoxide

(DMSO) and filtered through a 0.22-µm membrane.

The extraction solvent chosen in some cases was ethanol, which is generally

recognized as safe. Raw leaves (100 g) were oven dried at 75°C and pulverized, and then

immersed in ethanol solution (100 mL) for 2 days. The residues were re-extracted with ethanol

solution (100 mL) for a further 3 days. The combined extract solutions were concentrated using

a rotary evaporator under vacuum. The residues were evaporated to dryness under nitrogen gas

and freeze-dried. The dried extracts were dissolved in ethanol and filtered through a 0.22-µm membrane.

Purification of the active compound

The ether extract was fractionated with a silica gel column chromatography (60×5 mm i.d., 70–230 mesh) and eluted with *n*-hexane-ethyl acetate (25%→50%→75%→100% ethyl acetate, each 3 mL). Twelve fractions were collected in 1 mL volumes. The fractions were evaporated to dryness under nitrogen gas. The residues were dissolved in DMSO.

Separation of the ether extract was also performed by high-pressure liquid chromatography (HPLC) with UV detection using a YMC-Pack Pro C18 column (150×4.6 mm i.d., with 5 µm particle size) (YMC, Kyoto, Japan) using a flow-rate of 1 mL/min, at a temperature of 40°C. Mobile-phase A consisted of 99% (v/v) distilled water, 1% (v/v) acetonitrile, and 0.1% (v/v) trifluoroacetic acid (TFA). Mobile-phase B consisted of 99% (v/v) acetonitrile, 1% (v/v) distilled water, and 0.1% (v/v) TFA. The injected volume was 50 µL and the detection was performed at a wavelength of 300 nm. The eluates were collected in volumes of 1 mL for 1 min each. The fractions were evaporated to dryness under nitrogen gas and freeze-dried. The residues were dissolved in DMSO.

Nuclear magnetic resonance (NMR) and mass spectrometry (MS)

The structure of the isolated compound was chemically identified by NMR and mass spectrometry. NMR spectra were recorded using an Avance II 800US^2 spectrometer (Bruker BioSpin, Rheinstetten, Germany) at 800 MHz in DMSO-d_6. Chemical shifts for ^{1}H- and

^{13}C-NMR were referenced to tetramethylsilane (0.00 ppm). Assignments were made via

^{1}H-NMR, ^{13}C-NMR, distortionless enhancement by polarization transfer (DEPT), total

correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC) and

heteronuclear multiple-bond correlation (HMBC). High-resolution electron impact (EI)-MS

analysis was performed on a JEOL JMS-600H mass spectrometer (Tokyo, Japan).

Evaluation of cell viability

The cell viability of PC12 cells was determined by

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The culture

medium was replaced with serum-free medium containing 0.5 mg / mL MTT tetrazolium salt

(Nacalai Tesque, Kyoto, Japan) and incubated at 37°C for 30 min. The medium was removed

and the cells were scraped and solubilized into a 2-propanol solution. Aliquots were transferred

to a 96-well plate and the absorbance was measured at 570 nm. The viability of the cultures was

expressed as a percentage of the absorbance measured in control cells.

Small interfering RNA (siRNA) sequences and transfection

siRNAs were purchased from Invitrogen. The siRNA sequence targeting Nrf2 #1 was

5′-UUAAGACACUGUAACUCGGGAAUGG-3′ and Nrf2 #2 was

5′-UUUAAGUGGCCCAAGUCUUGCUCCA-3′. Stealth™ RNAi Negative Control Medium

GC Duplex #2 (Invitrogen) was used as a negative control siRNA. PC12 cells in 35 mm culture

dishes were transfected with each siRNA (200 pmol) using 7.5 µL Lipofectamine 2000

according to the manufacturer's protocol. The medium was changed after 9 h and cultures were

incubated for further 48 h.

Real-time PCR analysis

Total RNA was extracted using a FastPure® RNA kit (Takara, Shiga, Japan). Total RNA was reverse-transcribed using a PrimeScript® RT-PCR kit (Takara). Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara). The final solution contained 0.4 µM primers and 2 µL of cDNA in a total volume of 25 µL. The protocol involved denaturation at 95°C for 30 sec, and amplification (40 cycles: 95°C for 5 sec, 60°C for 30 sec). The primer sequences were Nrf2 forward 5′- GAGACGGCCATGACTGAT-3′, Nrf2 reverse 5′-GTGAGGGGATCGATGAGTAA-3′, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5′-ATGGGAAGCTGGTCATCAAC-3′, and GAPDH reverse 5′-GATCTCGCTCCTGGAAGATG-3′. The mRNA levels of tested genes were quantified using standard curves generated by serially diluted reference samples. The relative levels of Nrf2 mRNA were analyzed by normalizing with GAPDH mRNA expression.

Western blotting

Treated cells were washed twice with cold Tris-buffered saline, harvested using a cell scraper and lysed in buffer containing Tris (20 mM, pH 7.0), sodium β-glycerophosphate (25 mM), ethylene glycol tetraacetic acid (2 mM), Triton X-100 (1%), phenylmethylsulfonyl fluoride (1 mM), aprotinin (1%), dithiothreitol (2 mM) and vanadate (1 mM) on ice. Lysates were centrifuged at 17,000 g for 30 min at 4°C. After normalization of protein concentrations, supernatants were mixed in equal amounts with a sample loading buffer. After denaturation by

boiling at 100°C for 5 min, samples were loaded onto a SDS-polyacrylamide gel, separated

electrophoretically, and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford,

MA, USA). The membranes were incubated for 1 h with Tris-buffered saline containing 0.1%

Tween 20 and 5% dehydrated skim milk to block nonspecific binding. Subsequently, the

membranes were probed with primary antibody [anti-phospho-p38 MAPK (#4631, 1:1,000

dilution, Cell Signaling Technology, Danvers, MA, USA), anti-p38 MAPK (#9212, 1:1,000

dilution, Cell Signaling Technology), anti-phospho-Akt (#9271, 1:1,000 dilution, Cell Signaling

Technology), anti-Akt (#9272, 1:1,000 dilution, Cell Signaling Technology), anti-γ-GCS

(#RB-1697, 1:1,000 dilution, Neomarkers, Fremont, CA), HO-1 (#SPA-895, 1:100,000 dilution,

Stressgen, Victoria, Canada), anti-β-actin (#A1978, 1:20,000 dilution, Sigma)] and with

horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution, GE Healthcare,

Waukesha, WI, USA) for 1 h. The membrane-bound secondary antibody was detected with an

enhanced chemiluminescence detection system (ECL, GE Healthcare, Buckinghamshire, UK).

The band intensities were analyzed with computer software, ImageJ 1.33u (National Institute for

Health, Bethesda, MD, USA).

Measurement of intracellular reduced glutathione (GSH) levels

The levels of GSH were determined using monochlorobimane (MCB, Sigma), which

forms a fluorescent conjugate together with GSH. PC12 cells were incubated with

Krebs–Ringer–HEPES buffer (125 mM NaCl, 4.8 mM KCl, 25 mM HEPES, 1.2 mM MgSO$_4$,

1.2 mM KH$_2$PO$_4$, 5.6 mM glucose, 2.2 mM CaCl$_2$, pH7.4) containing MCB (50 µM) for 30

min, and lysed in Triton X-100 (1%). The fluorescence intensity of the lysates was measured

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using a spectrofluorometer (Ex 355 nm, Em 460 nm).

Detection of intracellular ROS

Levels of intracellular ROS were measured by flow cytometry and microscopic analysis as the fluorescence of 2′,7′-dichlorofluorescein (DCF) and ethidium (ETH), which are the oxidation products of 2′,7′-dichlorodihydrofluorescein (H_2DCF) and dihydroethidium (DHE). H_2DCF is more sensitive to hydrogen peroxide and hydroxyl radicals than to superoxide anions, whereas DHE is particularly sensitive to superoxide anions. H_2DCFDA (Molecular Probes, Eugene, OR, USA) is a diacetylated form of H_2DCF that is freely membrane permeable and enters the cells. After entering the cells, the diacetyl groups of H_2DCFDA are cleaved by intracellular esterases and the resulting H_2DCF can be oxidized to highly fluorescent DCF by ROS. DHE (Sigma) is a lipophilic cell-permeable dye that can undergo oxidation to red fluorescent ETH in the presence of ROS. ETH then binds irreversibly to the double-stranded DNA causing amplification of the red fluorescent signal.

For flow cytometry, treated cells were incubated for 30 min with the probe (H_2DCFDA: 30 µM, and DHE: 10 µM) at 37°C. A FACScan (Becton-Dickinson, Rutherford, NJ, USA) flow cytometer, equipped with a 488-nm argon ion laser and supplied with the Cell Quest software, was applied to measure ROS levels in the cells. Signals were obtained using a 530-nm bandpass filter (FL-1 channel) for DCF and a 585-nm bandpass filter (FL-2 channel) for ETH. Each determination was based on the mean fluorescence intensity of 1,000 cells. For microscopic analysis, cells were incubated with H_2DCFDA (10 µM) and a cell-permeable nuclear indicator Hoechst 33342 (100 µg/mL; Molecular Probes) for 30 min at 37°C. The cells

were rinsed twice with phenol red-free DMEM, and then treated with 6-OHDA. Fluorescence was visualized using an Olympus IX81 inverted microscope and Metamorph software.

For flow cytometry, H_2DCFDA was used at 30 μM, a concentration that permitted the quantification of baseline fluorescence as well as peak fluorescence without saturation. For microscopic analysis, however, the images at peak fluorescence were saturated because of a limited dynamic range. It was therefore necessary to reduce the concentration of H_2DCFDA to 10 μM. At this concentration, peak images were not saturated, but baseline levels were too low to be quantified with precision.

Statistics

The statistical significance of the differences between three or more groups was analyzed with a one-way analysis of variance (ANOVA) and post hoc multiple comparison using Turkey's test, unless otherwise stated. Statistical significance was defined as $p < 0.05$. Data are expressed as the mean ± standard error of the mean (SEM).

Results

Comparison of ARE activity between the extracts of fruits and vegetables

To evaluate the effects of fruit and vegetable extracts on the cellular defense response against oxidative stress, we established rat PC12 stable reporter cell lines expressing the luciferase coding sequences controlled by ARE from the rat NQO 1 gene. At first, PC12 reporter cells were treated with the ether extracts of fruits and vegetables for 9 h. The ether extracts of cranberry (juice) and green perilla (raw leaves) significantly increased luciferase activity (Fig. 1A), although cell death was not apparent at this time point. At the same concentration, native PC12 cells were treated with the ether extracts of fruits and vegetables for 48 h. The ether extracts of cranberry (juice), green perilla (raw leaves) and tossa jute (raw leaves) significantly decreased cell viability (Fig. 1B). Because the ether extract of cranberry, in particular, exhibited a potent cytotoxic effect, further luciferase assay-guided chromatographic separation was carried out on the ether extract of green perilla. The ether extracts of green perilla increased luciferase activity in a concentration-dependent manner (Fig. 1C). An ethanol extract (25–100%) of green perilla (raw leaves) was evaluated for ARE-dependent transcriptional activity. The higher the ethanol concentration was, the higher the luciferase activity of the ethanol extract (Fig. 1D).

Bioactivity-guided purification of the ARE activator from green perilla

The ether extract of green perilla (raw leaves) was subjected to a silica gel column chromatography using *n*-hexane-ethyl acetate (25%→50%→75%→100% ethyl acetate) to separate 12 fractions; among them, fraction 8 exhibited significantly increased luciferase activity (Fig. 2A). The ether extract of green perilla (raw leaves) was also subjected to reversed-phase HPLC with a C18 column and a water/acetonitrile solvent system (mobile-phase A, 99:1;

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mobile-phase B, 1:99) containing 0.1% TFA under a gradient of 1–100% mobile-phase B for 30

min (Fig. 2B). Fraction 18 exhibited significantly increased luciferase activity (Fig. 2C). The

eluted active fraction 18 was collected and subjected to the second purification step. In the

second purification by HPLC under an isocratic condition of 30% of mobile-phase B,

significantly increased luciferase activities were observed in fractions 34–36 (Fig. 2D and E). An

aqueous extract after ether/water extraction of green perilla was also subjected to reversed-phase

HPLC under a gradient of 1–100% mobile-phase B for 30 min, but there were no active

fractions (data not shown).

Structure elucidation of the ARE activator from green perilla

The active fractions 34–36 at the second purification step were collected and subjected

to the third preparative HPLC with a water/acetonitrile solvent system (mobile-phase A, water;

mobile-phase B, acetonitrile) under a gradient of 40–45% of mobile-phase B for 10 min. The

main peak collected showed a single band in thin layer chromatography (TLC) analysis (data

not shown). By this method, 11 mg of this compound was obtained from 100 g fresh weight of

green perilla leaves. Furthermore, high-resolution MS analysis demonstrated that the molecular

formula of this compound was $C_{17}H_{16}O_5$ (observed: 300.0985, calculated mass: 300.0988).

NMR studies (^{1}H, ^{13}C, DEPT, TOCSY, HMQC, and HMBC) revealed that the chemical

structure of this compound was 2′,3′-dihydroxy-4′,6′-dimethoxychalcone (DDC; Fig. 3A),

previously reported by Ichino et al. [25]. DDC has been previously isolated from leaves of

Uvaria dulcis and whole plants of *Sarcandra hainanensis*, but pharmacological properties of the

compound have not yet been investigated [26] and [27].

Activation of ARE-dependent transcription by DDC

DDC was synthesized from 2′-hydroxy-4′,6′-dimethoxyacetophenone and

trans-cinnamoyl chloride by Friedel–Crafts reaction [25]. ^{1}H-NMR analysis demonstrated that

synthetic DDC showed identical chemical shifts and peak pattern with the isolated compound

(data not shown). Synthetic DDC was tested for its effect on ARE activity. Treatment of PC12

reporter cells with DDC resulted in a significant increase in luciferase activity. The effect of

DDC on ARE activity was concentration-dependent and peaked at 9–12 h (Fig. 4A and B). At

concentrations ($< 30 \mu M$) that had enough ARE activity, exposure to DDC for 48 h exhibited no

cytotoxic effect (Fig. 4C). A structure–activity relationship of chalcone derivatives (Fig. 3B and

C) was investigated to clarify the structural requirements for DDC. Chalcone (compound **1**)

without any substituted group and cinnamaldehyde (compound **8**), a substructure of chalcone,

exhibited a slight increase in luciferase activity. Among the tested chalcone derivatives,

dimethoxychalcone, including DDC, compound **6**, and compound **7** had comparatively potent

activity (Fig. 4D). Sulforaphane ($3 \mu M$), a well-known ARE activator, was used as a positive

control. Treatment of PC12 cells with sulforaphane ($10 \mu M$ and more) for 48 h exhibited

cytotoxicity (data not shown). Thus, we successfully identified DDC as the active compound

responsible for the induction of ARE-dependent transcription in green perilla.

Regulation of DDC-induced ARE-dependent transcriptional activation

In general, binding of Nrf2 to ARE is necessary for antioxidant gene expression. To

confirm the involvement of Nrf2 in DDC-induced ARE activation, PC12 cells were transfected

with siRNAs against Nrf2. Quantitative real-time PCR analysis demonstrated that the mRNA level of Nrf2 was partially decreased by Nrf2 siRNA (Fig. 5A). The increase in luciferase activity by DDC was also partially suppressed by transfection of siRNA directed against Nrf2 (Fig. 5B). It has been reported that the phosphorylation of Nrf2 plays an important role in nuclear translocation and transcriptional activation through ARE. Our previous report showed that nuclear translocation of Nrf2 in PC12 cells was mediated via phosphorylation by protein kinases [28]. After treatment with DDC, phosphorylated p38 mitogen-activated protein kinase (MAPK) and phosphorylated Akt were transiently increased around the 1-h time point (Fig. 5C and D). Furthermore, the increase in luciferase activity by DDC was suppressed by SB203580, an inhibitor of p38 MAPK, and LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K), in an additive manner (Fig. 5E).

Induction of antioxidant enzymes by DDC

The Nrf2-ARE pathway is critical in mediating the induction of γ-GCS, a rate-limiting enzyme in glutathione synthesis, NQO1, and HO-1. The induction of these antioxidant enzymes by DDC in PC12 cells was investigated. Treatment with DDC for 24 h increased γ-GCS protein level, GSH content, NQO1 activity and HO-1 protein level at concentrations that are comparable with those that increased ARE-dependent luciferase activity (Fig. 6A, C, E, and G). The effects of DDC on γ-GCS protein level, GSH content, and NQO1 activity were suppressed by co-treatment with SB203580 (Fig. 6B, D, and F). The effect of DDC on HO-1 protein level was not suppressed by either SB203580 or LY294002 (Fig. 6H and I), but decreased by a combination of SB203580 and LY294002 (Fig. 6J).

Cytoprotective effect of DDC against 6-OHDA-induced cytotoxicity

6-OHDA is extensively used to induce cell death through oxidative processes in different cell types, including human neuroblastoma SH-SY5Y cells, PC12 cells and rat ventral mesencephalic dopaminergic neurons [28], [29], and [30]. To test the protective effect of DDC on oxidative stress, PC12 cells were challenged with 6-OHDA. Pre-treatment with DDC for 24 h alone provided protection against 6-OHDA-induced cytotoxicity to a similar extent as pre-treatment followed by co-treatment during the toxin exposure. However, co-treatment with DDC slightly exacerbated 6-OHDA-induced cytotoxicity (Fig. 7A). Pre-treatment with DDC provided protection at concentrations that are comparable with those that increased ARE-dependent luciferase activity (Fig. 7B). The cytoprotective effect of DDC was suppressed by SB203580 (Fig. 7C). To confirm the indirect antioxidant properties of DDC, intracellular ROS levels were measured using oxidant-sensitive dyes (H_2DCFDA and DHE). Pre-treatment with DDC for 24 h suppressed the increase in intracellular ROS levels induced by 6-OHDA (Fig. 7D-F). In particular, DDC decreased the basal level of DCF-sensitive ROS (Fig. 7E). Therefore, our results suggest that the cytoprotection of DDC is mediated by indirect antioxidant properties via ARE activation.

Discussion

In this study, we compared the effects of various fruit and vegetable ether extracts on ARE-dependent transcriptional activation. Green perilla extract exhibited a high level of ARE activity. Furthermore, we identified DDC from the extract as an active ingredient responsible for the ARE activity. Our data demonstrated that DDC enhanced the induction of antioxidant enzymes through activation of the Nrf2-ARE pathway and provided protective action against 6-OHDA-induced cytotoxicity.

Perilla (*Perilla frutescens*; Labiatae) is an annual herbaceous plant native to Asia. There are two forms, the green form (green perilla) and the red form (red perilla), which differ in their accumulation of anthocyanins [31]. It has been reported that perilla leaves and seeds contained the anti-allergic, anti-inflammatory, anti-carcinogenic, and anti-human immunodeficiency virus substances, such as rosmarinic acid and α-linolenic acid [32], [33], [34], and [35]. Because of their high abundance of antioxidant polyphenolic compounds, perilla leaf water extracts possessed potent radical scavenging activity [36]. In addition, we show that green perilla leaf ether extract potentiates the cellular defense system against oxidative stress. Although we showed that red perilla juice failed to increase ARE activity, red perilla leaf ether extract possessed ARE activation potency, although the effect was not so potent as green perilla leaf extract (data not shown). Considering that ARE activity of green perilla ethanol extracts increased in proportion to ethanol concentration and that green perilla water extract failed to increase ARE activity, it is suggested that the active ingredients responsible for ARE activation in perilla have comparatively low solubility in water.

Chalcone is a class of flavonoid compounds that are widely biosynthesized in plants

and exhibits a basic structure of two benzene rings linked through an α, β-unsaturated carbonyl group. Chalcone derivatives possess a diverse spectrum of biological activities, including anti-oxidative, anti-inflammatory, anti-cancer, and immunosuppressive potential [37], [38], and [39]. DDC has been previously isolated and identified from leaves of *Uvaria dulcis* and whole plants of *Sarcandra hainanensis* [26] and [27]. However, to our knowledge, there are no reports on the existence of DDC in green perilla, which is a dietary vegetable. Furthermore, the present study is the first report on the biological activity of DDC.

The present study provides the first evidence that DDC intensively induces Nrf2-dependent ARE activation. The activation of the Nrf2-ARE pathway by various electrophiles and compounds that are acceptors for Michael reactions is attributed to changes in redox environment and/or direct cysteine modification in Keap1 [40]. The α, β-unsaturated carbonyl moiety in the chalcone skeleton provides the basis for the reaction of a Michael acceptor with a nucleophile. Previous study found that the α, β-unsaturated carbonyl moiety was crucial for nucleophilic addition reactions with thiols and for increasing intracellular ROS levels [41]. Talalay *et al*. [42] demonstrated that many α, β-unsaturated carbonyl compounds induced antioxidant enzymes and that the potency of inducers paralleled their efficiency in Michael reactions. Although the α, β-unsaturated carbonyl moiety in chalcone plays a basic role in ARE activation, the present structure–activity relationship study revealed that the substituted groups on chalcone were of critical importance for potency. In addition, depending on cell type and inducer, multiple signaling kinases have been reported to regulate the Nrf2-ARE pathway, which include p38 MAPK, extracellular signal-regulated kinase, c-jun NH_2-terminal kinase, PI3K and protein kinase C [43]. Our findings indicate that p38 MAPK pathway and PI3K/Akt

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pathway additively participated in ARE activation induced by DDC. Further studies are needed to elucidate the mechanisms of Nrf2-dependent ARE activation induced by DDC.

Our data demonstrated that DDC provided protection against 6-OHDA-induced cytotoxicity. The cytoprotection of DDC against 6-OHDA toxicity did not require co-treatment but pre-treatment before the toxin challenge. The mechanisms underlying the effectiveness of DDC against 6-OHDA cytotoxicity are not completely known, but the indirect antioxidative properties of DDC could explain its protective effect. We showed that DDC was able to completely reverse the drastic increase in ROS formation induced by 6-OHDA. DDC increased antioxidative enzymes, such as γ-GCS, NQO1, and HO-1 in a concentration-dependent manner. The cytoprotection induced by DDC in accordance with the induction of γ-GCS, GSH, and NQO1 was abolished by inhibition of p38 MAPK pathway. These results suggest that the cytoprotection of DDC may be mediated by the antioxidant enzymes up-regulated by p38 MAPK-dependent ARE activation. 6-OHDA is rapidly oxidized by molecular oxygen to form superoxide anions, hydrogen peroxide, and the corresponding p-quinone, which contribute to the cytotoxicity [27]. GSH scavenge ROS and interact with 6-OHDA-oxidized products, forming glutathionyl conjugates [44]. NQO1 catalyzes the two-electron reduction of quinones to hydroquinones, which prevents the formation of highly reactive semiquinones. In addition, catalase and superoxide dismutase have been reported to be up-regulated by ARE activation [45]. Therefore, antioxidant enzymes regulated via the Nrf2-ARE pathway may comprehensively participate in the cytoprotection of DDC. Elevation of HO-1 levels by DDC was not abolished by inhibition of either p38 MAPK pathway or PI3K/Akt pathway. However, inhibition of both p38 MAPK pathway and PI3K/Akt pathway suppressed the induction of

HO-1 levels. Therefore, these pathways may serve as a compensatory mechanism for HO-1 upregulation. In addition, since the treatment with DDC (30 µM) in the presence of both inhibitors of p38 MAPK and PI3K for 24 h was injurious to cells (data not shown), elevation of HO-1 levels by DDC seems to play an important role in defensive mechanisms against DDC-induced cell damage.

In summary, we succeeded in isolating and identifying the Nrf2-ARE activator from green perilla ether extract. DDC was found to induce the expression of various antioxidant proteins, suppress intracellular ROS formation, and thereby enhance cellular resistance to 6-OHDA-induced cytotoxicity. Previous studies have described the nuclear localization of Nrf2 in patients with Parkinson disease and Nrf2-mediated neuroprotection in models of Parkinson disease [46], [47], and [48]. Therefore, DDC may have the potential to be developed as a therapeutic agent aimed at reducing or preventing cell death in Parkinson disease. Therefore, we propose that indirect as well as direct antioxidant activity of dietary fruits and vegetables are beneficial to human health.

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Figure legends

Fig.1 Effects of fruit and vegetable extracts on ARE activity. A: Effects of ether extracts of fruits and vegetables on ARE activity. PC12 reporter cells were treated with ether extracts for 9 h. The concentrations of each extract were: peach (juice), 100 µL/mL; apple (juice), 50 µL/mL; strawberry (juice), 100 µL/mL; cranberry (juice), 100 µL/mL; raspberry (juice), 100 µL/mL; satsuma mandarin (juice), 20 µL/mL; green perilla (raw leaves), 50 mg/mL; tossa jute (raw leaves), 25 mg/mL; crown daisy (raw leaves), 20 mg/mL; celery (juice), 100 µL/mL; parsley (juice), 50 µL/mL; red perilla (juice), 100 µL/mL; crown daisy (juice), 100 µL/mL. B: Effects of ether extracts of fruits and vegetables on cell viability. Native PC12 cells were treated with ether extracts for 48 h. C: Concentration-dependent effect of ether extract of green perilla on ARE activity. PC12 reporter cells were treated with ether extracts of green perilla for 9 h. D: Effects of ethanol extracts of green perilla on ARE activity. PC12 reporter cells were treated with ethanol extracts of green perilla for 9 h. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, compared with control.

Fig. 2 Chromatographic purification of active fraction from ether extract of green perilla. A: Effects of fractions separated by silica gel column chromatography on ARE activity. B: HPLC-UV (300 nm) chromatogram of the first purification step of green perilla ether extract. C: Effects of first HPLC fractions of green perilla ether extract on ARE activity. D: HPLC-UV (300 nm) chromatogram of the second purification step of green perilla ether extract. E: Effects of second HPLC fractions of green perilla ether extract on ARE activity. PC12 reporter cells were treated with each fraction of green perilla for 9 h. $^{\#\#\#}p < 0.001$, compared with control.

Fig. 3 Chemical structures of chalcone derivatives. A: 2′,3′-Dihydroxy-4′,6′-dimethoxychalcone (DDC). B: Chalcone derivatives. C: Cinnamaldehyde.

Fig. 4 Effect of synthetic DDC on ARE activity. A: Concentration-dependent effect of DDC on ARE activity. PC12 reporter cells were treated with DDC (3–30 µM) for 9 h. B: Time-dependent effect of DDC on ARE activity. PC12 reporter cells were treated with DDC (30 µM) for the indicated periods. Statistical analyses were performed using two-way ANOVA and post hoc multiple comparison using Bonferroni test. C: Effect of DDC on cell viability. Native PC12 cells were treated with DDC (1–100 µM) for 48 h. D: Effects of chalcone derivatives on ARE activity. PC12 reporter cells were treated with chalcone derivatives (30 µM) or sulforaphane (3 µM) for 9 h. [###]$p < 0.001$, compared with control.

Fig. 5 Mechanisms of ARE activation induced by DDC. A: Effect of Nrf2 siRNA on Nrf2 mRNA expression. Native PC12 cells were incubated for 48 h after transfection with siRNA against Nrf2. B: Effect of Nrf2 siRNA on ARE activation induced by DDC. PC12 reporter cells were incubated for 48 h after transfection, and then were treated with DDC (30 µM) for a further 12 h. C,D: Effect of DDC on phosphorylation of p38 MAPK and Akt. Native PC12 cells were treated with DDC (30 µM) for the indicated periods. E: Effects of p38 MAPK and PI3K inhibitors on ARE activation induced by DDC. PC12 reporter cells were treated with DDC (30 µM) in the presence or absence of SB203580 and LY294002 (10–30 µM) for 9 h. [###]$p < 0.001$ compared with control. [***]$p < 0.001$ compared with DDC alone.

Fig. 6 Effect of DDC on ARE-regulated antioxidant proteins. A, C, E, G: Effect of DDC on

γ-GCS protein level (A), GSH content (C), NQO1 activity (E), and HO-1 protein level (G).

Native PC12 cells were treated with DDC (3–30 µM) for 24 h. B, D, F: Effect of p38 MAPK

inhibitor on DDC-induced elevation of γ-GCS protein level (B), GSH content (D), and NQO1

activity (F). Native PC12 cells were treated with DDC (30 µM) in the presence or absence of

SB203580 (10–30 µM) for 24 h. H-J: Effects of p38 MAPK and PI3K inhibitors on

DDC-induced elevation of HO-1 protein level. Native PC12 cells were treated with DDC (30

µM) in the presence or absence of SB203580 and LY294002 (10–30 µM) for 24 h. $^{\#\#}p < 0.01$,

$^{\#\#\#}p < 0.001$, compared with control. $^{***}p < 0.001$ compared with DDC alone.

Fig. 7 Effect of DDC on 6-OHDA-induced cytotoxicity. A: Time course study on the effect of

DDC on 6-OHDA toxicity. Native PC12 cells were exposed to 6-OHDA (200 µM) for 24 h

with or without pre-treatment for 24 h and co-treatment with DDC (30 µM). B:

Concentration-dependent effect of DDC on 6-OHDA toxicity. Native PC12 cells were treated

with DDC (3–30 µM) for 24 h prior to 6-OHDA exposure, and then exposed to 6-OHDA (200

µM) for 24 h. C: Effect of p38 MAPK inhibitor on DDC-induced cytoprotection against

6-OHDA toxicity. Native PC12 cells were treated with DDC (30 µM) in the presence or

absence of SB203580 (3–30 µM) for 24 h prior to 6-OHDA exposure, and then were exposed to

6-OHDA (200 µM) for 24 h. D-F: Effect of DDC on 6-OHDA-induced ROS formation. D:

Representative fluorescence microscope images from DCF fluorescence. Native PC12 cells

were pretreated with DDC (30 µM) for 24 h, incubated with H_2DCFDA for 30 min, and

exposed to 6-OHDA (200 µM) for 1 h. *Scale bar* = 50 µm. E,F: Analysis of DCF and ETH

fluorescence by flow cytometry. Native PC12 cells were treated with DDC (3–30 µM) for 24 h prior to 6-OHDA exposure, and then were exposed to 6-OHDA (200 µM) for 3 h. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with 6-OHDA alone. $^{\dagger\dagger\dagger}p < 0.001$ compared with 6-OHDA+DDC.