A Thesis for the Degree of Master of Science

Protective Effects of 5-(3′,4′-Dihydroxylphenyl)-ɣ-valerolactone, a major Metabolite of Cocoa Procyanidins, on Hydrogen Peroxide-induced Apoptosis in Primary Cortical Neurons

DHPV의 산화적스트레스에 의한

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ABSTRACT

Procyanidins are the most abundant phytochemicals in the dietary food and responsible for the health effects of cocoa. However due to low absorption of procyanidins, recently researchers are focusing on the simple metabolites of cocoa that are catalyzed by intestinal microbiota and exert health effects. The aim of this study is to figure out the neuroprotective effect of 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (DHPV), a min metabolite of cocoa procyanidins, against hydrogen peroxide-induced oxidative stress in primary cortical neurons. Oxidative stress is strongly associated with many diseases such as neurodegenerative disorders, cancers and cardiovascular diseases and oxidative stress is induced by reactive oxygen species such as hydrogen peroxide (H_2O_2).

In this study, I identified the protective effects of DHPV on oxidative

neuronal death. Results showed that H_2O_2-induced nuclear condensation in apoptotic neurons was inhibited by pre-treatment with DHPV. Pre-treatment with DHPV prevented the H_2O_2-induced decrease of anti-apoptotic protein, Bcl-2 and inhibited H_2O_2-induced cleavage of caspase-3 and poly(ADP-ribose) polymerase. I also found that DHPV induced the expression of NADPH:quinone oxidoreductase 1 (NQO1) and enhanced nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), a principal antioxidant transcription factor in neuronal cells, demonstrating that DHPV protects neurons from H_2O_2-induced apoptosis by up-regulation of phase Ⅱ antioxidant enzymes.

Keywords: Cocoa procyanidins; DHPV; Microbial metabolite; Apoptosis; Primary cortical neurons; oxidative stress; NQO1, Nrf2

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CONTENTS

Ⅰ. INTRODUCTION

Cocoa is a dietary source rich in phytochemicals and presents a higher antioxidant activity than well-known antioxidant foods such as fruits, green tea and red wine [1]. Cocoa consists of numerous nonflavonoid and flavonoid phenols and flavan-3-ols are the dominant phytochemicals in cocoa [2-4]. Several studies suggested that cocoa has beneficial effects on brain health [5, 6]. It has been reported that (-)-epicatechin prevented the stroke damage via activating nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant pathway and consumption of flavanol-rich cocoa exerted promising effects on cognitive tasks [7, 8]. Cocoa procyanidins also attenuated the neuronal cell death induced by hydrogen peroxide and 4-hydroxynonenal (HNE) through MAPK pathway in PC12 cells [9, 10].

Studies on the metabolomics suggest that despite the abundance in nature and its promising effects on central nervous system, flavan-3-ols exerts low bioavailability in our body [11]. Flavan-3-ols consist of monomeric catechins and epicatechins, and polymeric procyanidins [12, 13]. Bioavailability of flavan-3-ols differs greatly on the degree of polymerization and intestinal absorption of polymeric procyanidins is limited due to its large molecular size and high hydrophilicity [14, 15]. Polymeric procyanidins reach the colon and catabolized by the gut microbiota into small molecules that could easily absorbed and exert health effects [11]. Among many metabolites, DHPV is a major metabolite produced from the microbial metabolism of cocoa procyanidins [16-18]. Procyanidins convert to diphenylpropan-2-ols through the breakdown of the heterocyclic C ring and with cleavage of A ring and lactonization, diphenylpropan-2-ols result in the formation of 5-

(3', 4'-dihydroxyphenyl)-γ-valerolactone (DHPV) [16, 19]. Recent studies reported that DHPV has antioxidative, antiproliferative, and anti-inflammatory activities [20-22], however the biological properties related to the brain have not been clarified.

Neurodegenerative diseases refer to the aging of brain and strongly related to oxidative stress. Oxidative stress-induced neuronal cell death and brain dysfunction is the major cause of neurodegenerative diseases [23]. Alzheimer's diseases is a brain disorder characterized by the accumulation of amyloid β (Aβ) peptide and generation of reactive oxygen species (ROS) [24, 25]. Neuronal cells are particularly more susceptible to oxidative stress than cells in other organs [26]. Brain accounts for only 2% of total body weight, however energy metabolism in brain is highly an aerobic process that consumes nearly 20% of total oxygen consumption in our body. Therefore, relatively high amount of

reactive oxygen species is produced which is harmful to neurons. On the contrary, brain is relatively lack of antioxidant enzymes such as catalase [27]. In addition, neuronal cells contain high concentration of unsaturated fatty acids and metal ions that catalyze the radical formation [26, 28]. Hydrogen peroxide (H_2O_2) is a main component of ROS and cause detrimental damage to DNA, protein, and lipids [29]. Therefore, H_2O_2 is widely used to investigate the mechanisms of oxidative stress in neurodegenerative disorders.

In this study, I identified the neuroprotective effects of DHPV, a major metabolite of cocoa procyanidins and its mechanisms. I used primary cortical neurons to test the effect of DHPV in similar physiological conditions to actual neuronal cells in brain and H_2O_2 to induce apoptosis. I confirmed that H_2O_2-induced neuronal cell death was mediated by Bcl-2, caspase-3, Poly(ADP-ribose) polymerase (PARP),

ROS generation, NQO1 up-regulation and Nrf2 activation. Our results show that DHPV protects neuronal cells from H_2O_2-induced apoptosis by regulating the Nrf2 cellular antioxidant defense system.

Ⅱ. MATERIALS AND METHODS

1. Chemicals and reagents

5-(3',4'-dihydroxyphenyl)-gamma-valerolactone(DHPV) was purchased from Chemieliva phamaceutical company (China). Poly-D-lysine (PDL) was purchased from Merk Millipore (Billerica, MA USA). 4,6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Burlingame, CA, USA). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was obtained from Invitrogen (Carlsbad, CA, USA). Thiazolyl blue tetrazolium bromide (MTT) was purchased from USB (Cleaveland, OH, USA). Mg2+- and Ca2+-free Hanks' balanced salt solution (HBSS), neurobasal medium, B27 supplement, L-glutamine (200 mM), trypsin-EDTA and penicillin/streptomycin were obtained from Gibco BRL (Carlsbad, CA, USA). Hydrogen peroxide (H_2O_2) was

purchased from Biosesang (Seongnam, Korea). Anti-Bcl-2, anti-PARP, anti-NQO1, anti-b-actin, anti-laminB and anti-Nrf2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl-xL, anti-HO-1 and anti-caspase-3 antibodies were obtained from Cell signaling Technology (Danvers, MA, USA). All other chemicals used were of analytic grade.

2. Primary neuronal culture

Primary cortical neurons were obtained from pregnant ICR mice at embryonic day 14 (Orient Bio, Seongnam, Korea). Cerebral cortices were isolated from the embryos and placed in ice-cold Mg2+- and Ca2+-free Hanks' balanced salt solution (HBSS). Collected cerebral cortices were centrifuged (300g, 2min), dissociated using 0.05% trypsin in HBSS for 8 min at 37℃ and washed twice with HBSS, once with Neurobasal

medium. Then cells were resuspended in Neurobasal medium supplemented with 2% B27, 2 mM L-glutamine, 1% penicillin/streptomycin and filtered by 40μm cell strainer. The isolated cortical neurons were plated on poly-d-lysine coated plates and incubated in a 37℃ humidified atmosphere of 5% CO2. Culture medium was changed a day after cortical neurons were seeded and every two days with Neurobasal medium supplemented with 2% B27, 2 mM L-glutamine, 1% penicillin/streptomycin.

3. MTT assay

MTT assay was used to determine the cell viability. MTT assay measure the amount of insoluble purple formazan which is formed by mitochondrial dehydrogenases in live cells. Primary cortical neurons (5 x 10^5 cells/well in 96-well plates) were treated with H_2O_2 concentration up

to 100 μM and DHPV concentration up to 80 μM for 24h to determine the toxicity of H_2O_2 and DHPV. Also cells were incubated at 37℃ with 50 μM H_2O_2 for 24h with or without pretreatment with DHPV and then treated with the 1 mg/ml MTT solution for 2h without light exposure. The dark purple formazan crystals formed in intact cells were dissolved with 150 μl DMSO, and the absorbance at 570nm was measured with a microplate reader. The results are expressed as the percentage relative to the control.

4. Tunel assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-fluorescein nick end labeling (TUNEL) assay was conducted to determine whether the DHPV pretreatment has protective effects on apoptosis in primary cortical neurons. TUNEL assay detects an important

characteristic of apoptosis, DNA fragmentation by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using Terminal Deoxynucleotidyl Transferase (TdT). Primary cortical neurons (5 x 10^5 cells/chamber in Lab-Tek chamber slide) were incubated at 37℃ with 50 μM H_2O_2 for 24h with or without pretreatment with DHPV and neurons were fixed with 4% para-formaldehyde for 25 minutes at 4℃. After washing with PBS, neurons were permeabilized with 0.2% Triton X-100 solution in PBS for 5minutes. TUNEL assay was conducted according to the manufacturer's protocol (Promega). The stained apoptotic neurons were observed using a fluorescence microscope.

5. Western blot analysis

Primary cortical neurons (4 x 10^6 cells/3 ml in a 6-cm dish) were incubated at 37℃ with 50 μM H_2O_2 for 24h with or without pretreatment

with DHPV and then washed twice with PBS and lysed using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Beverly, MA, USA). The harvested cells were incubated on ice for 30 min vortexing every 10 minutes and centrifuged at 14000 rpm for 10 min. The supernatant was separated and stored at –70℃. Cytoplasmic and nuclear cell lysates were isolated by NE-PER kit (Thermo scientific, Waltham, MA, USA) according to the manufacturer's protocol. The protein concentration was determined using a protein assay reagent (Bio-rad, Hercules, CA, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride trasnfer membrane (Merk Millipore, Billerica, MA USA). The transferred membrane was blocked with 5% skim milk for 1h at room temperature and incubated with specific primary antibodies overnight at 4℃. After three washes with TBST (Tris-buffered saline with 0.1% Tween 20), blots were

incubated with horseradish peroxidase-conjugated secondary antibody in 5% skim milk at a 1:5000 dilution for 1h. Then washed with TBST three times again and the blots were developed using the enhanced chemiluminescence(ECL) detection kit (GE Healthcare, St, Giles, UK) according to the manufacturer's protocol. Densitometry analysis of bands was using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

6. Measurement of intracellular ROS level

Intracellular ROS level was measured by using the fluorescent probe DCFH-DA. DCFH-DA enters the cell and reacts with intracellular radicals, forming its fluorescent form, DCF. Primary cortical neurons were cultured at 37℃ in 96-well plate (5 x 10^5 cells/well) and preincubated with 5-40 μM DHPV for 20 min. After that 20 μM DCFH-

DA was loaded for 20 min and then exposed to 100 μM H_2O_2 for 10 min. Cells were examined at 529 nm with a fluorescence spectrophotometer (Infinite M200; Tecan Trading, Switzerland), with excitation at 495 nm.

7. Immunofluorescence

Primary cortical neurons were washed with PBS three times and fixed with 4% formaldehyde for 15 min and washed with PBS three times again. Fixed cells were permeabilized with 0.2% Triton X-100 for 5 min, washed with PBS and blocked in 10% normal goat serum for 1h at room temperature. Then cells were treated with primary antibody against Nrf2 in 10% normal goat serum at 4℃ overnight. After three times washing with PBS, a fluorescein isothiocyanate-conjugated secondary antibody diluted in 10% normal goat serum at a ratio of 1:500 was treated for 2h at room temperature. After that, nuclear staining was performed using DAPI

staining and cells were observed by using a Zeiss LSM700 confocal microscopy.

8. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of triplicate experiments. Comparisons between groups were analyzed by Student's t-test and $p<0.05$ was considered as significant difference.

Ⅲ. RESULTS

1. DHPV concentration up to 80 μM has no toxic effect on primary cortical neurons.

To examine the cytotoxicity of DHPV and H_2O_2 on primary cortical neurons, I first investigated the cell viability change on various concentrations of DHPV and H_2O_2. I treated neurons with DHPV or H_2O_2 only for 24h in a wide range of concentrations. Neuronal cells treated with DHPV concentration up to 80μM showed no significant difference in cell viability (Fig. 2A). Exposure of primary cortical neurons to H_2O_2 (6.25 – 100 μM) for 24h resulted in decrease of cell survival rate (Fig. 2B). I determined 50 μM H_2O_2 as an inducer at which cell viability decreased nearly 50% compared to control.

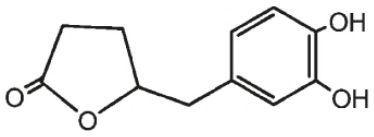


Figure 1. Chemical structure of DHPV.

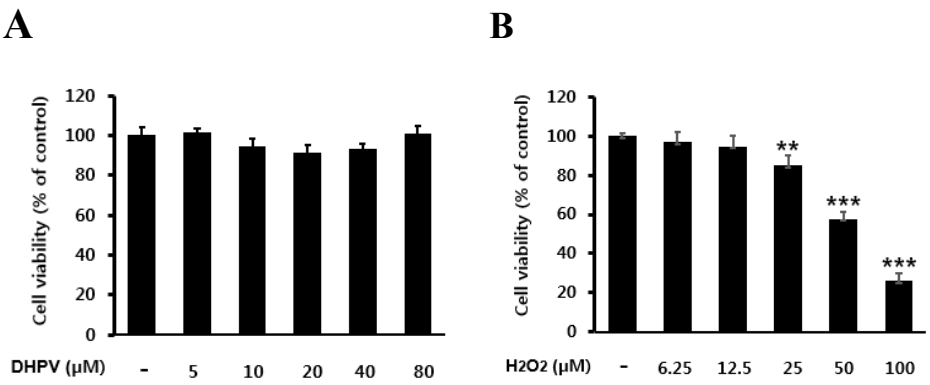


Figure 2. The cytotoxic effect of DHPV and H_2O_2 on primary cortical neurons. Cells were incubated in the presence of various concentrations of DHPV or H_2O_2 for 24h. (A, B) In separate experiments, cell viability of neurons incubated with DHPV or H_2O_2 was measured by MTT assay. Cell viability was expressed as a relative percentage of control. Data are

expressed as means ± SE (n=3). ** $p<0.01$, *** $p<0.001$, in comparison to the control.

2. DHPV inhibits H_2O_2-induced apoptosis in primary cortical neurons.

The protective effect of DHPV on H_2O_2-induced neuronal cell death was examined by using microscopy and MTT assay. Primary cortical neurons were pretreated with DHPV at 5, 20 μM for 1h followed by 50 μM H_2O_2 for 24h (Fig. 3). Morphological change observed by microscopy showed that treatment of 50 μM H_2O_2 for 24h induced cleaved-neuronal dendrites and condensed cell bodies, however, neurons pretreated with DHPV at 5, 20 μM for 1h showed similar morphology to that of control (Fig. 3A). Also, the viability of cells incubated with 50 μM H_2O_2 for 24h was decreased to 47.5±1.74% of the control value, and the viability of cells with DHPV (5, 10, 20 μM) were increased to 58.9±8.5, 68.5±7.7, 76.2±4.6% in a dose-dependent manner (Fig. 3B).

The neuroprotective effect of DHPV was further confirmed by

Tunel assay. Nuclear DNA fragmentation is a major hallmark of apoptotic cell death and to determine the type of cell death, I conducted a Tunel assay. Tunel data show that treatment of 50μM H_2O_2 for 24h dramatically increased DNA fragmentation and this was inhibited by treatment of DHPV (Fig. 3C and D). Preincubation of cells with DHPV at 5, 20 μM for 1h decreased apoptotic cells significantly (Fig. 3C and D). Quantification of tunel-positive cells in percentage of total cells was shown in Fig. 3D. Thus, H_2O_2-induced apoptosis in primary cortical neurons is attenuated by DHPV.

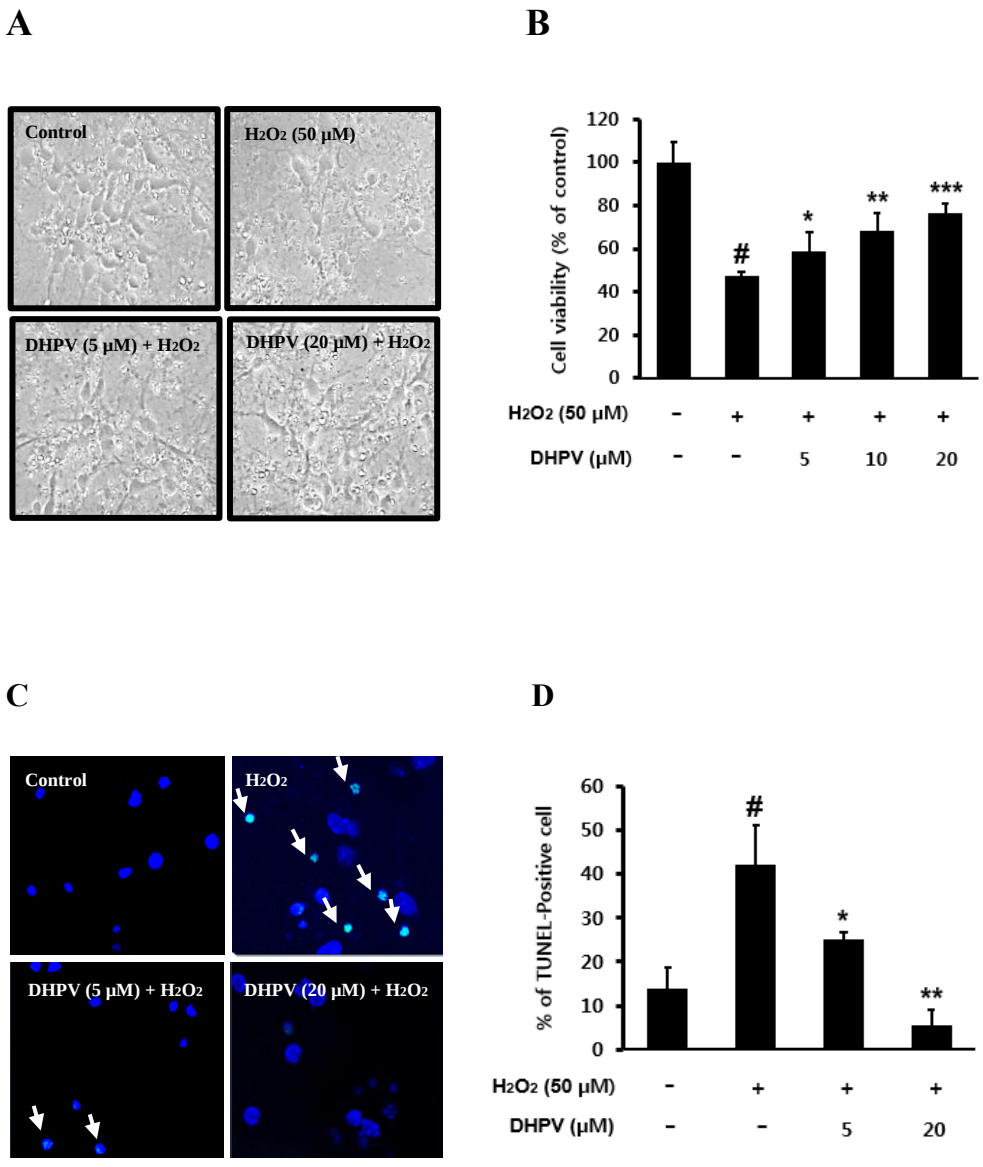


Figure 3. Neuroprotective effects of DHPV on H_2O_2-induced apoptosis in primary cortical neurons. (A) Cell morphology change was observed

under a microscope. DHPV attenuates H_2O_2-induced neuronal cell death. (B) The viability of neurons was examined by MTT assay. (C) The DNA fragmentation of apoptotic cells were determined by Tunel assay under confocal microscope as describe in Material and methods. The white arrows show DNA fragmentation of cells. (D) Quantitative data on DNA fragmentation was expressed as the number of Tunel-positive cells per total cells. Data are means ± SEM values from three independent experiments. # $p<0.05$, compared with control cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with cells only treated with H_2O_2.

3. DHPV attenuates H_2O_2-induced down-regulation of Bcl-2 and cleavage of caspase-3 and pro-PARP.

Apoptosis is regulated by the balance between anti-apoptotic and pro-apoptotic proteins. Among them, Bcl-2 family are major anti-apoptosis proteins that inhibit the activation of the caspase-3 cascade and apoptosis by regulating the mitochondrial membrane potential [30]. To elucidate the molecular mechanism underlying the neuroprotective effects of DHPV on H_2O_2-induced cell death, I measured the intracellular concentrations of Bcl-2 and Bcl-xL in primary cortical neurons. Neuronal cells treated with 50 μM H_2O_2 for 24h exhibited down-regulation of Bcl-2 but the effects of H_2O_2 were attenuated by pretreatment with DHPV at 5, 20 μM for 1h. There was no significant change in Bcl-xL (Fig. 4A and B).

The mitochondrial dysfunction is closely related to oxidative stress and apoptosis [31]. When oxidative stress increase, caspase-3 is

cleaved and become an active form [32]. Caspase-3, a zymogen that exist as an inactive form, is a main pro-apoptotic protein that mediate the apoptosis. Pretreatment with DHPV at 5, 20 μM before exposure to 50 μM H_2O_2 for 24h prevented H_2O_2-induced cleavage of caspase dramatically (Fig. 4A and C). In turn, activated caspase-3 cleaves poly (ADP-ribose) polymerase (PARP), an important functional protein responsible for DNA repair [33]. When cellular stress damages DNA, PARP adds poly (ADP-ribose) polymers [34]. Neurons exposed to 50 μM H_2O_2 for 24h increased the level of cleaved-PARP and it was attenuated by pretreatment of DHPV at 5, 20 μM for 1h (Fig. 4A and D).

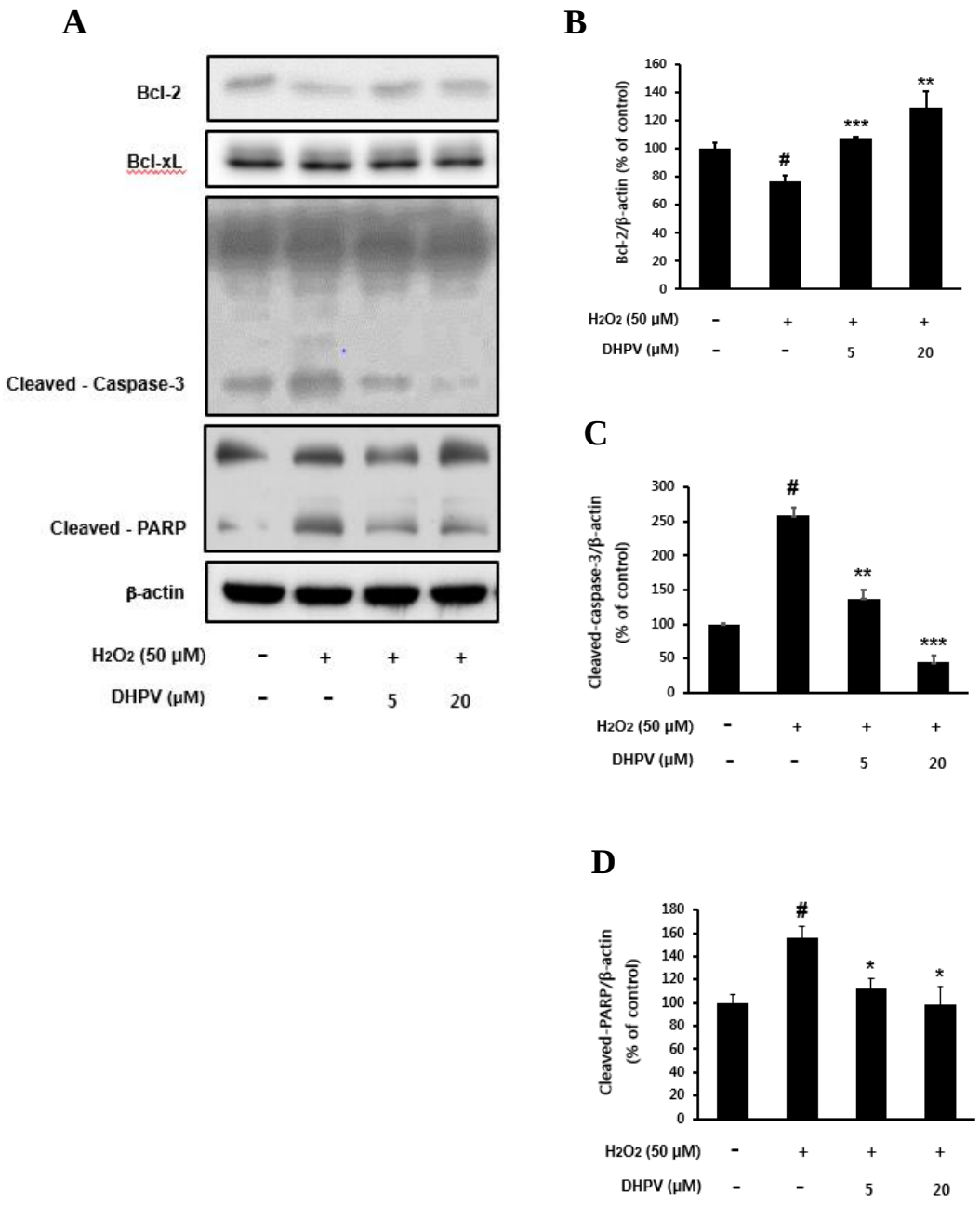

A
Bcl-2
Bcl-xL
Cleaved - Caspase-3
Cleaved - PARP
β-actin
H2O2 (50 μM) - + + +
DHPV (μM) - - 5 20
B
Bcl-2/β-actin (% of control)
160
140
120
100
80
60
40
20
0
#
***
**
H2O2 (50 μM) - + + +
DHPV (μM) - - 5 20
C
Cleaved-caspase-3/β-actin (% of control)
300
250
200
150
100
50
0
#
**
***
H2O2 (50 μM) - + + +
DHPV (μM) - - 5 20
D
Cleaved-PARP/β-actin (% of control)
180
160
140
120
100
80
60
40
20
0
#
*
*
H2O2 (50 μM) - + + +
DHPV (μM) - - 5 20

Figure 4. Effects of DHPV on H_2O_2-induced down-regulation of Bcl-2 and Bcl-xL, activation of caspase-3 and cleavage of PARP in primary cortical neurons. Cells were preincubated with DHPV (5, 20 μM) for 1h and then exposed to 50 μM H_2O_2 for 24h. (A) The protein levels of Bcl-2, Bcl-xL, cleaved-caspase-3, cleaved-PARP were determined by Western blot analysis. β-actin was also measured to confirm equal protein loading. The relative protein expression of (B) Bcl-2/β-actin, (C) Cleaved-caspase-3/β-actin and (D) Cleaved-PARP/β-actin was indicated by densitometry. Data are expressed as means ± SEM of three independent experiments. # $p<0.05$, compared with control cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with cells only treated with H_2O_2.

4. DHPV reduces intracellular ROS level by up-regulating the expression of NQO1.

To identify whether the neuroprotective effect of DHPV against H_2O_2-induced apoptosis is an antioxidative effect, I measured the intracellular ROS level using DCFH-DA method. Treatment of H_2O_2 resulted in increase in intracellular ROS level, compared to control and pretreatment with DHPV at 5, 20 μM for 1h inhibited H_2O_2-induced ROS accumulation (Fig. 5A).

Antioxidant enzymes is a well-known cellular defense system and by regulating the expression level of antioxidant enzymes, cell counteracts the oxidative stress. I hypothesized that the neuroprotective effects of DHPV on H_2O_2-induced apoptosis in primary cortical neurons may be due to the NQO1, HO-1 antioxidant enzymes and investigated the intracellular protein expression level of NQO1, HO-1. Primary cortical neurons pretreated with DHPV at 5, 20 μM for 1h followed by 50 μM

H_2O_2 for 1h significantly increased the NQO1 protein level in dose-dependent manner (Fig. 5B and C). There was no change in HO-1 (Fig. 5B).

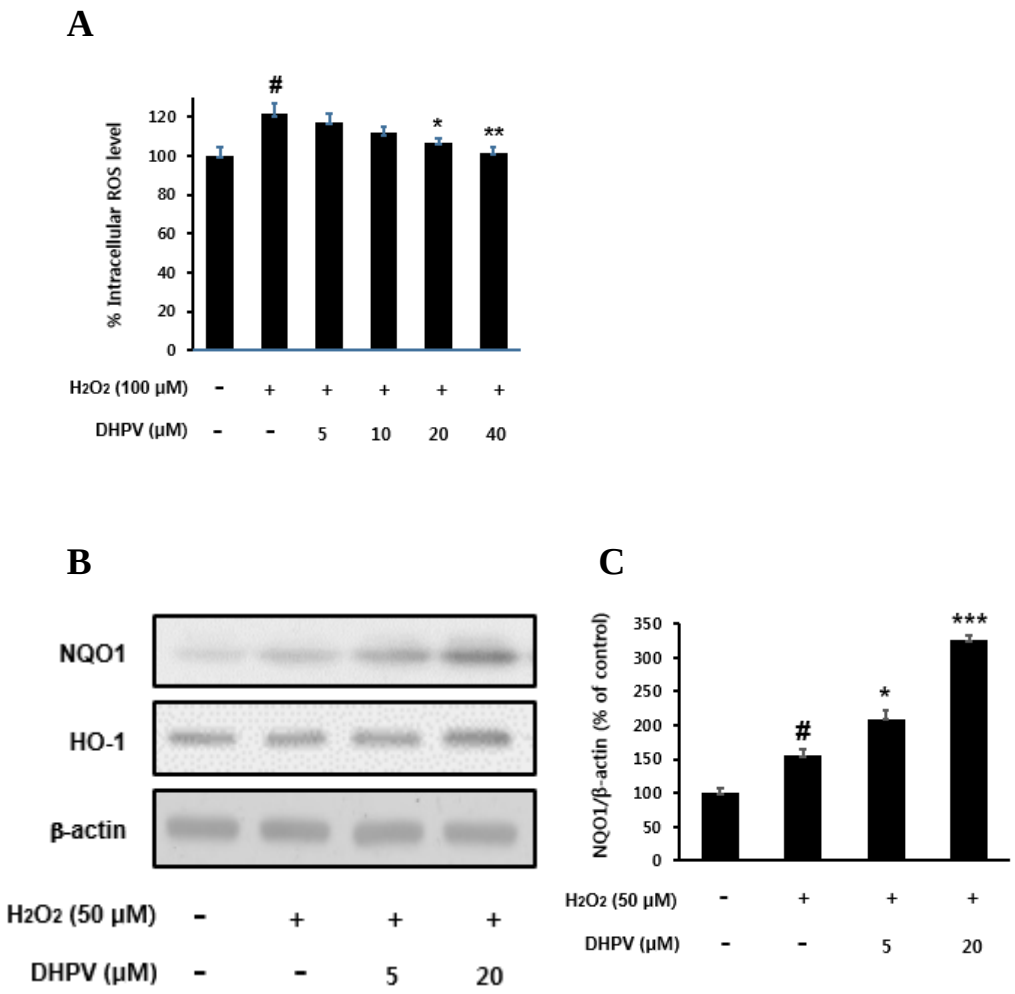


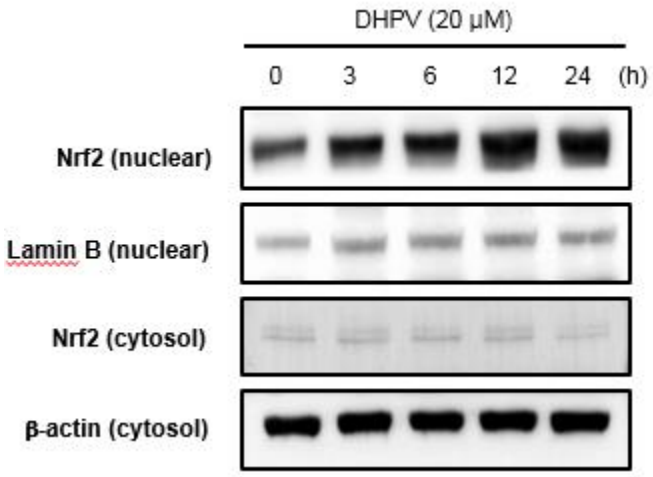
Figure 5. Antioxidative effect of DHPV on H_2O_2-induced ROS accumulation and upregulation of NQO1. (A) Cells were pretreated with different doses of DHPV for 20 min and then incubated with DCFDA

and H_2O_2 subsequently. DCF fluorescence was measured under fluorescence spectrophotometer (Ex. 495nm, Em.529nm). (B) Cells were preincubated with DHPV at 5, 20 μM for 1h before exposure to 50 μM H_2O_2 for 24h. The protein levels of NQO1 and HO-1 was measured by Western blot analysis. β-actin was also measured to confirm equal protein loading. (C) The ratio of NQO1/β-actin was indicated by densitometry. Data are expressed as means ± SEM of three independent experiments. # $p<0.05$, compared with control cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with cells only treated with H_2O_2.

5. DHPV up-regulate the activation of major antioxidant transcriptional factor, Nrf2.

To find out the underlying molecular mechanism in the upregulation of antioxidant enzymes, I examined a key antioxidant transcriptional factor, nuclear factor erythroid 2-related factor 2 (Nrf2). First, I determined the protein level of Nrf2 in nuclear and cytosol after treatment with DHPV at 20 μM for 0, 3, 6, 12, 24h . As time goes, Nrf2 accumulation in nuclear increased and there was no significant change in cytosolic protein level of Nrf2 (Fig. 6A). To confirm the Nrf2 translocation from cytosol to nuclear, I conducted the immunofluorescence using confocal microscopy. Treatment with DHPV at 20 μM induced Nrf2 nuclear translocation in a time-dependent manner (Fig. 6B).

A


DHPV (20 μM)
0
3
6
12
24
(h)
Nrf2 (nuclear)
Lamin B (nuclear)
Nrf2 (cytosol)
β-actin (cytosol)

B

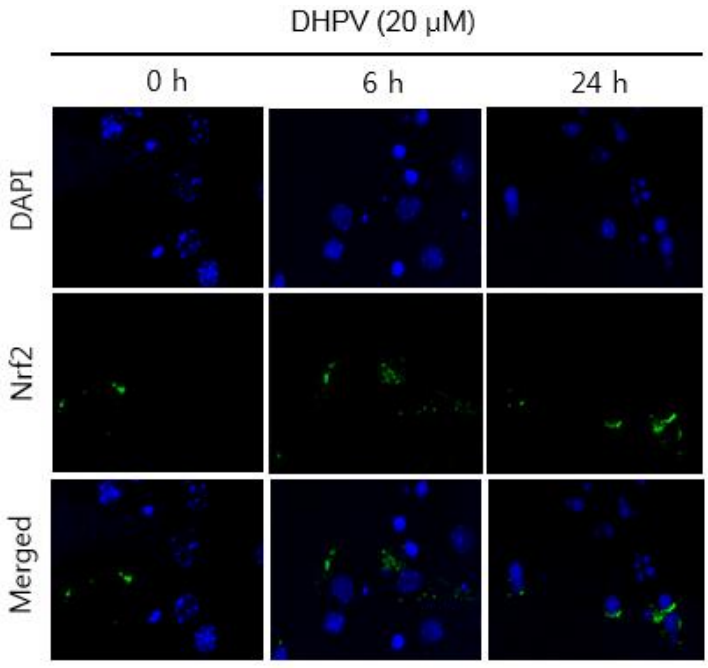

DHPV (20 μM)
0 h
6 h
24 h
DAPI
Nrf2
Merged

Figure 6. Effect of DHPV on Nrf2 activation in primary cortical neurons. Neurons were incubated with DHPV at 20 μM for indicated time periods. (A) The protein levels of cytosolic and nuclear Nrf2 were measured by Western blot analysis. Lamin B and β-actin was also measured to confirm equal protein loading. (B) Nrf2 nuclear translocation at 6, 24h after treatment with DHPV was confirmed by immunofluorescence method using confocal microscopy.

Ⅳ. DISCUSSION

Polyphenols have received substantial attentions for its potential effects on cancer, cardiovascular diseases and neurodegenerative diseases. Cocoa is a polyphenol-rich food and procyanidins are the most abundant polyphenol in cocoa [35]. Previous studies indicate that after consumption of procyanidin-rich foods, DHPV was detected in human blood plasma [17, 36]. Metabolomics on flavanols identified that DHPV is detected from the plasma membrane after intake of procyanidin-rich cocoa and purified procyanidin dimers and demonstrated that DHPV is a major metabolite of cocoa procyanidins [16, 37]. Furthermore, DHPV has a higher antioxidant activities and radical scavenging capacities than vitamin C and catechin [21]

In this study, I elucidated that DHPV, a major metabolite of cocoa procyanidins, has a neuroprotective effect against H_2O_2-induced

apoptosis via Nrf2 antioxidant pathway. I confirmed that treatment of 50 μM H_2O_2 induced the decrease of Bcl-2 protein expression level and increase of cleaved-caspase-3 and cleaved-PARP protein expression levels. DHPV inhibited the down-regulation of anti-apoptotic protein Bcl-2 and cleavage of key pro-apoptotic proteins caspase-3 and pro-PARP. I also checked increase of ROS generation and DNA fragmentation, important characteristics of apoptosis induced by oxidative stress.

Treatment with DHPV increased the expression of NQO1, phase Ⅱ detoxification enzyme via activating Nrf2-ARE pathway which partially contributes to the neuroprotective effect of DHPV. NQO1 is a well-known phase Ⅱ detoxification enzyme that converts toxic quinone to hydroquinone and excretes by conjugated with glucuronic acid or glutathione [38]. NQO1 also catalyzes the reduction of two electrons and

provides electrons for recycling of antioxidants and energy metabolism [39]. When oxidative stress increases and mitochondria functions are impaired, NQO1 is up-regulated and protects the cells as a cellular defense mechanism [40].

Nrf2 transcription factor is known as a regulator of numerous antioxidant enzymes with antioxidant response element [41]. Therefore Nrf2 pathway is critical for maintaining the homeostasis of cells and organisms to various oxidative stressors and electrophiles. Under basal conditions, Nrf2 is tightly bound to cysteine residues of Keap1 and undergoes ubiquitination and protein degradation by Cul3-based E3 ligase [42]. Upon electrophilic and oxidative stressors, Keap1 is oxidatively modified and binding between Nrf2 and Keap1 is loosen and translocate from cytosol to nucleus where transcription of cytoprotective genes occurs [42]. Results of this study indicate that DHPV may acts as

an electrophilic agent and promotes the translocation of Nrf2 to nucleus.

In neuroscience, permeability across the blood-brain barrier (BBB) is important criteria on research of potential therapeutic agent for neurodegenerative diseases. DHPV has low molecular weight (MW 208) and has number of H-bonding less than 8, which satisfies the criteria of BBB permeability [20, 43]. Therefore, DHPV is considered to be able to pass through the BBB. In further study, it is necessary to check the concentration of DHPV in brain in vivo models. In addition, comparison on the neuroprotective effects between the DHPV and its potential precursors such as (+)-catechin, (-)-epicatechin, procyanidin A1, A2, B1 and B2 is needed to elucidate more precise mechanism of neuroprotective effect of cocoa acting on the brain.

Present study is a first report to demonstrate the potent neuroprotective effect of DHPV on H_2O_2-induced apoptosis. DHPV

inhibits the down-regulation of Bcl-2, cleavage of caspase-3 and pro-PARP and increase the protein level of NQO1 via activation of Nrf2 pathway. These results suggest that DHPV could be a potential therapeutic candidate for the prevention of neurodegenerative diseases.

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Ⅵ. 국문 초록

카카오에는 폴리페놀 성분이 다량 함유되어 있으며 프로시아니딘은 카카오에 가장 많이 들어있는 폴리페놀 성분 중 하나이다. 카카오 프로시아니딘이 뇌에 미치는 긍정적인 효과에 대해 많은 선행연구들이 이뤄졌으며 최근 연구 결과에 따르면 프로시아니딘을 섭취했을 시 소장에서 흡수되지 않고 대장으로 내려가 장내미생물에 의해 대사체로 분해된다는 것이 밝혀졌다. 따라서 카카오 프로시아니딘이 대사되어 생성되는 대사체가 실질적으로 뇌에 이로운 영향을 미칠 것이라고 생각하였고 본 연구에서는 카카오 프로시아니딘의 주요 대사체인 DHPV의 뇌신경세포 보호 효능을 학인하고 그 작용기작을 규명하였다.

본 연구의 목적은 DHPV가 일차신경세포에서 항산화 효능을 통해 H2O2에 의해 유도된 신경세포사멸을 억제하는 효능을 규명하는 것으로, 퇴행성 뇌신경계질환, 암, 심혈관계 질환의 대표적인 원인이 되는 산화적스트레스를 유발하는 인듀서로 H2O2를 사용하였다. 연구 결과, DHPV를 전 처리한 군

에서 H_2O_2에 의해 유도된 핵 응축현상이 억제되었으며 세포사멸 억제 단백질인 Bcl-2 단백질 발현량이 증가하였다. 또한 세포사멸을 나타내는 대표적인 단백질인 caspase-3, pro-poly(ADP-ribose) polymerase의 cleaved-form이 줄어드는 것을 확인하였다. 신경세포사멸 억제 원인을 밝히고자 항산화효소 단백질양의 변화를 확인하였고, NADPH:quinone oxidoreductase 1 (NQO1)이 DHPV를 처리한 군에서 유의적으로 증가하는 것을 알 수 있었다. NQO1을 조절하는 상위인자를 밝히기 위해 대표적인 항산화효소 전사인자인 nuclear factor erythroid 2-related factor 2 (Nrf2)의 세포 내 발현양을 측정하였고, 그 결과 Nrf2가 세포질에서 핵으로 이동하는 것을 확인하였다.

본 연구 결과를 통해 DHPV가 세포 내 항산화 기작을 활성화시켜 산화적 스트레스에 의한 뇌신경세포 손상을 보호하는 효능이 있음을 확인하였다.