Cocoa procyanidins protect PC12 cells from hydrogen-peroxide-induced apoptosis by inhibiting activation of p38 MAPK and JNK

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Abstract

Oxidative stress induced by reactive oxygen species has been strongly associated with the pathogenesis of neurodegenerative disorders, including Alzheimer’s disease. In this study, we investigated the possible protective effects of a cocoa procyanidin fraction (CPF) and procyanidin B2 (epicatechin-(4β-8)-epicatechin) – a major polyphenol in cocoa – against apoptosis of PC12 rat pheochromocytoma (PC12) cells induced by hydrogen peroxide (H2O2). CPF (1 and 5 µg/ml) and procyanidin B2 (1 and 5 µM) reduced PC12 cell death caused by H2O2, as determined by MTT and trypan blue exclusion assays. CPF and procyanidin B2 attenuated the H2O2-induced fragmentation of nucleus and DNA in PC12 cells. Western blot data demonstrated that H2O2 induced cleavage of poly(ADP-ribose)polymerase (PARP), downregulated Bcl-XL and Bcl-2 in PC12 cells. Pretreatment with CPF or procyanidin B2 before H2O2 treatment diminished PARP cleavage and increased Bcl-XL and Bcl-2 in PC12 cells. Furthermore, H2O2-induced rapid and significant phosphorylation of c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), and both of these effects were attenuated by CPF or procyanidin B2 treatment. These results suggest that the protective effects of CPF and procyanidin B2 against H2O2-induced apoptosis involve inhibiting the downregulation of Bcl-XL and Bcl-2 expression through blocking the activation of JNK and p38 MAPK.

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1. Introduction

Alzheimer’s disease (AD) is the most common form of dementia characterized by progressive impairment in cognitive faculty and behavior [1], and is an age-related and irreversible brain disorder that develops over a period of years. Although the exact cause of AD remains unknown, accumulating studies provide evidence that oxidative stress and injury induced by free radicals are contributing risk factors. Amyloid-β peptide (Aβ), which is considered to have a role in the development and progress of AD, has the ability to generate reactive oxygen species (ROS) [2]. Oxidative stress is associated with cell apoptosis in the pathogenesis of neurodegenerative disorders, including AD [3]. ROS such as hydrogen peroxide (H2O2), the superoxide anion, and the hydroxyl radical are mediators of oxidative stress. As the major component of ROS, H2O2 is known to cause lipid peroxidation and DNA damage in cells [4].

H2O2-induced apoptosis has been associated with alteration in antiapoptosis- and apoptosis-related proteins. Bcl-XL and Bcl-2 are major antiapoptosis protein regulating cell survival and cell death [5], and is also reported to protect H2O2-induced apoptosis in neurons [6]. Caspases are cysteine proteases that are implicated to be key executors in cell death, which are divided into effector caspases (−3, −6, and −7) and initiator caspases (−2, −8, −9, and −10) [7,8]. Caspase-3 is reportedly activated by H2O2 as an effector of apoptotic cell death [9]. The activated caspase-3 cleaves the poly(ADP-ribose) polymerase (PARP),
which is known to be a hallmark of apoptosis [10]. Members of the mitogen-activated protein kinase (MAPK) family participate in the many signaling pathways associated with stress. Members of each major MAPK subfamily – the extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAPK – have been shown to be activated in response to proinflammatory and other stress signals [11]. JNK and p38 MAPK are apoptosis factors mediated by oxidative stress [12]. A previous study revealed that abnormal levels of phosphorylated JNK and p38 MAPK in the brain of AD patients is associated with oxidative stress [13]. These studies suggest that MAPKs modulate neurodegenerative disease.

Cocoa is abundant in phenolic phytochemicals and exhibits higher antioxidative activity than red wine, green tea, and black tea [14]. Several studies suggested that cocoa exerts health-promoting effects, such as inhibiting carcinogenesis [15], antioxidant effects, and protective effects against neurotoxicity [16]. A recent clinical study showed the potential benefits of the consumption of flavanol-rich cocoa on cognitive tasks and brain perfusion [17,18]. Procyanidin B2 (epicatechin-(4β-8)-epicatechin) (Fig. 1) is one of the polyphenols that is widespread in nature and in processed foodstuffs such as cocoa, chocolate, red wine, and fruit juice [19]. Several studies have shown that procyanidin B2 can exert various physiological effects, such as antioxidant activity [20], antitumor effect [21], and protection against DNA damage induced by Fe(II)/H2O2 [22]. In addition, procyanidin B2 is present in human plasma after the consumption of flavanol-rich cocoa or chocolate [23]. However, the underlying mechanism of the neuroprotective effects of cocoa and procyanidin remains to be clarified.

The PC12 rat pheochromocytoma (PC12) cell line constitutes a useful model for studying apoptosis mechanisms, including its prevention [24]. H2O2 is used to trigger apoptosis in PC12 cells [4,25]. An increased production of ROS or a poor antioxidant defense mechanism leads to physiological dysfunction and progressive damage in the cell. Here we report that a cocoa extract and procyanidin B2 protect PC12 cells from oxidative stress through blocking the phosphorylation of JNK and p38 MAPK.

### 2. Materials and methods

#### 2.1. Sample preparation

Cocoa procyanidins were extracted from commercially available cocoa powder as described previously [15]. Briefly, commercial cocoa powder (50 g) was extracted with 500 ml of 50% (v/v) aqueous ethanol under reflux for 6 h. After the extraction, the solution was filtered twice to collect the extract. The collected cocoa extract was loaded onto a styrene-based adsorption resin column (60 mm × 450 mm; HP-20, Mitsubishi, Japan), washed with 20% (v/v) aq. ethanol, and then eluted with 60% (v/v) aq. ethanol. The eluted cocoa procyanidin fraction (CPF) was concentrated at 40 or 50 °C under reduced pressure, then frozen and dried.

#### 2.2. Chemicals

Procyanidin B2 was purchased from Funakoshi (Funakoshi, Japan). H2O2 was obtained from Junsei Chemical (Tokyo, Japan). 4,6-Diamidino-2-phenylindole (DAPI) and trypan blue solution (0.4%) were purchased from Sigma Chemical (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, horse serum, and a penicillin/streptomycin mixture were obtained from Gibco BRL (Grand Island, NY, USA). Anti-PARP, anti-Bcl-2, anti-JNK, and anti-p38 MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An anti-β-actin antibody was purchased from Sigma Chemical. Anti-Bcl-XL, anti-cleaved-PARP, anti-cleaved-caspase-3, and anti-phosphorylated-JNK antibodies were purchased from Cell Signaling (Beverly, MA, USA). An anti-phosphorylated-p38 MAPK antibody was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). SB203580 was purchased from Calbiochem (San Diego, CA, USA). All other chemicals used were of analytical grade.

#### 2.3. Cell culture

PC12 cells kindly provided by Dr. Y.-J. Surh (Seoul National University) were grown in DMEM supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 0.1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 10% CO2 and 90% air. The SH-SY5Y cells purchased from KCLB (Korean cell line bank) were grown in DMEM supplemented with 10% fetal bovine serum, 0.1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

#### 2.4. MTT assay

The MTT assay provides a sensitive measurement of the normal metabolic status of cells, particularly that of mitochondria, which reflects early cellular redox changes. PC12 or SH-SY5Y cells (2 × 104 cells/well in 96-well plates) were incubated at 37 °C with 200 μM H2O2 for 24 h with or without pretreatment with CPF, procyanidin B2 or SB203580 and then treated with the MTT solution (final concentration, 1 mg/ml) for 2 h. The dark-blue formazan crystals formed in intact cells were dissolved in DMSO, and the absorbance at 570 nm was measured with a microplate reader. The results are expressed here as the percentage MTT reduction relative to the absorbance of control cells.

#### 2.5. Trypan blue exclusion assay

The trypan blue exclusion assay is based on trypan blue dye interacting with the cell if the membrane is damaged, since the chromophore is only excluded from viable cells. PC12 cells (104 cells/well in 6-well plates) were suspended after being incubated at 37 °C with 200 μM H2O2 for 24 h with or without pretreatment with CPF or procyanidin B2. After centrifugation at 600 × g for 6 min, cells were resuspended in 200 μl of a phosphate-buffered saline (PBS). The total suspension cell was mixed with 200 μl of 0.4% trypan blue staining solution for 5 min at room temperature. The cells were loaded into a hemocytometer, and those exhibiting dye uptake were counted under a microscope. The percentage of total cells stained was counted by scoring 150 cells.

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Fig. 1. Chemical structure of procyanidin B2.
2.6. DAPI staining assay

The fluorescent dye DAPI was used to detect the nuclear fragmentation that is a characteristic of apoptotic cells. PC12 cells (5 × 10^4 cells/well in 24-well plates) were incubated at 37 °C with 200 μM H_2O_2 for 24 h with or without pretreatment with CPF, procyanidin B2, or SB203580 and then washed with PBS and fixed with 70% ethanol for 20 min. The fixed cells were washed with PBS and stained with the DNA-specific fluorochrome DAPI (1 μg/ml). Following 10 min of incubation, the cells were washed with PBS, and the plates were observed under a fluorescence microscope (Olympus Optical, Japan).

2.7. DNA fragmentation analysis

An apoptotic cell is characterized by its unique ladder of nucleotide fragments in DNA-agarose gel electrophoresis. PC12 cells (1.6 × 10^6 cells/8 ml in an 8.5-cm dish) were incubated at 37 °C with 200 μM H_2O_2 for 24 h with or without pretreatment with CPF or procyanidin B2, and then washed and collected with ice-cold PBS and centrifuged at 200 × g for 10 min. Cellular DNA was isolated using a DNA isolation buffer (10 mM EDTA, 50 mM Tris–HCl (pH 8.0), 0.5% SDS, and 0.5 mg/ml Proteinase K) and then incubated for 4 h at 50 °C. After centrifugation at 1000 × g for 15 min, the supernatants were extracted with an equal volume of phenol, chloroform, and isoamyl alcohol. The DNA was then mixed with 4 M sodium chloride and 100% ethanol and stored at −70 °C overnight. Each DNA sample was loaded onto a 1.8% Tris–boric acid–EDTA agarose gel and electrophoresed at 100 V for 30 min.

2.8. Western blot analysis

PC12 cells (2 × 10^5 cells/4 ml in a 6-cm dish) were incubated at 37 °C with 200 μM H_2O_2 for 24 h with or without pretreatment with CPF or procyanidin B2, and then washed and collected with ice-cold PBS and centrifuged at 600 × g for 10 min. The cell pellet was resuspended in 100 μl of ice-cold lysis buffer (Cell Signaling) and incubated on ice for 30 min. After centrifugation at 1000 × g for 15 min, the supernatant was separated and stored at −70 °C. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins were separated on an SDS–polyacrylamide gel, and then transferred onto a polyvinylidene difluoride transfer membrane that was blocked with 5% skim milk containing 0.5 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween-20 for 2 h at room temperature. The membrane was subsequently incubated with the primary antibody. After three washes with TBST (Tris-buffered saline with 0.1% Tween-20), the blots were incubated with horseradish-peroxidase-conjugated secondary antibodies in TBST with 5% skim milk at a 1:5000 dilution for 2 h at room temperature. The blots were then again washed three times in TBST buffer. Blots were developed using the enhanced chemiluminescence (ECL) detection method by immersing them for 5 min in a mixture of ECL reagents A and B at the ratio 1:1 and exposing them to photographic film for a few minutes.

2.9. Statistical analysis

Where appropriate, data are expressed as mean ± S.D. values, and Student’s t-test was used for single comparisons. A probability value of p < 0.05 was used as the criterion for statistical significance.

3. Results

3.1. Inhibition of H_2O_2-induced PC12 cell death by CPF and procyanidin B2

We first examined the possible protective effects of CPF and procyanidin B2 on H_2O_2-induced PC12 cell death using the MTT reduction assay. Cells were pretreated with CPF or procyanidin B2 at the indicated concentrations for 30 min, and then further treated with 200 μM H_2O_2 for 24 h. The viability of cells incubated with 200 μM H_2O_2 for 24 h was 53.6 ± 5.3% of the control value, and this increased to 74.2 ± 7.3%, 83.5 ± 10.2%, 64.4 ± 10.7%, and 79.2 ± 10.8% when they were pretreated with CPF at 1 and 5 μg/ml or procyanidin B2 at 1 and 5 μM, respectively (Fig. 2A). The cytoprotective effects of CPF and procyanidin B2 were also verified by the trypan blue exclusion
assay. Compared with the control group, the viability of cells after exposure to 200 μM H₂O₂ for 24 h was 36.7 ± 2.2%, and this increased to 61.7 ± 3.7%, 81.1 ± 4.8%, 66.9 ± 4.0%, and 74.7 ± 4.4% for preincubation with CPF at 1 and 5 μg/ml or procyanidin B2 at 1 and 5 μM, respectively (Fig. 2B). The PC12 cells exposed to 5 μg/ml CPF or 5 μM procyanidin B2 only for 24 h did not exhibit significant difference compared with control (Fig. 2A and B). We further investigated the effects of CPF and procyanidin B2 on H₂O₂-induced SH-SY5Y cell death using the MTT reduction assay. The viability of cells exposed to 200 μM H₂O₂ for 24 h was 53.1 ± 1.4% and the viabilities of cell pretreated with CPF at 1 and 5 μg/ml or procyanidin B2 at 1 and 5 μM were increased in a significant manner to 64.9 ± 6.8%, 88.1 ± 1.9%, 66.8 ± 5.3% and 91.6 ± 3.2% of control value, respectively (Fig. 2C). The SH-SY5Y cells exposed to only CPF 5 μg/ml or procyanidin B2 5 μM for 24 h did not exhibit significant difference compared with control (Fig. 2C). Thus, H₂O₂ treatment reduced the viability of cells, and this was protected by CPF and procyanidin B2 in a dose-dependent manner.

3.2. Attenuation of H₂O₂-induced apoptosis by CPF and procyanidin B2

Apoptosis is morphologically characterized by nuclear disintegration and cleavage of DNA into 200-bp fragments. Two indices were applied to identify whether H₂O₂-induced cell
death through the induction of apoptosis: (1) nuclear condensation, as measured by fluorescence microscopy, and (2) DNA fragmentation, as measured by agarose gel electrophoresis. PC12 cells were preincubated for 30 min with CPF (1 and 5 μg/ml) or procyanidin B2 (1 and 5 μM), and then exposed to 200 μM H₂O₂ for 24 h (Fig. 3A). DAPI staining indicated that H₂O₂ induced nuclear condensation in a large proportion of the PC12 cells, and this was significantly decreased by pretreatment with 5 μg/ml CPF or 5 μM procyanidin B2 (Fig. 3A). The nuclear condensation of PC12 cells exposed to only CPF 5 μg/ml or procyanidin B2 5 μM for 24 h did not exhibit alteration compared with control group (Fig. 3A). DNA was extracted from cells treated with 200 μM H₂O₂ for 24 h and also from those pretreated for 30 min with 5 μg/ml CPF or 5 μM procyanidin B2 prior to treatment with 200 μM H₂O₂ for 24 h. These pretreatments dramatically decreased DNA fragmentation (Fig. 3B), indicating that CPF and procyanidin B2 protect against H₂O₂-induced apoptosis in PC12 cells.

3.3. Prevention of H₂O₂-induced cleavage of PARP and downregulation of Bcl-Xₐ and Bcl-2 by CPF and procyanidin B2

Cleavage of PARP is associated with the induction of apoptosis in neurons. Bcl-Xₐ and Bcl-2 are major antiapoptosis proteins which are downregulated by apoptosis. These proteins represent biochemical hallmarks of apoptosis, and hence were examined to elucidate the underlying molecular mechanism of the protective effects of CPF and procyanidin B2 on H₂O₂-induced cell death. We measured the intracellular concentrations of these proteins in PC12 cells which were pretreated with CPF (1 and 5 μg/ml) or procyanidin B2 (1 and 5 μM) for 30 min prior to treating them with 200 μM H₂O₂ for 24 h. The treatment with 200 μM H₂O₂ induced cleavage of PARP and downregulation of Bcl-Xₐ and Bcl-2 as determined by Western blot analysis. CPF (1 and 5 μg/ml) and procyanidin B2 (1 and 5 μM) inhibited H₂O₂-induced cleavage of PARP and downregulation of Bcl-Xₐ and Bcl-2 in a dose-dependent manner (Fig. 4A and B, respectively).

3.4. Inhibition of H₂O₂-induced activation of caspase-3 by CPF and procyanidin B2

Caspases are cysteine proteases that mediate cell death and present as proenzymes that are cleaved and activated during apoptosis. Caspase-3 has been shown to be an important regulator of apoptosis. Therefore, we assessed the appearance of the cleaved caspase-3 at 19 kDa by Western blot analysis. Cleavage of caspase-3 was evidently shown on cells treated with H₂O₂ for 24 h, and this was markedly reduced by pretreatment with CPF (1 and 5 μg/ml) (Fig. 5A) or procyanidin B2 (1 and 5 μM) (Fig. 5B).

3.5. Inhibition of H₂O₂-induced phosphorylation of JNK and p38 MAPK by CPF and procyanidin B2

Activation of MAPK have been implicated in the brains of AD patients [13]. It is reported that SP600125 and SB203580 – which are specific inhibitors of JNK and p38, respectively – prevent the PC12 cell death induced by H₂O₂ [26]. We confirmed that exposure of PC12 cells to 200 μM H₂O₂ induced the phosphorylation of JNK and p38 MAPK, and this was inhibited by pretreatment with CPF (1 and 5 μg/ml) or procyanidin B2 (1 and 5 μM) (Fig. 6A and B, respectively).

3.6. Inhibition of H₂O₂-induced PC12 cell death by SB203580

In previous study, we confirmed that SP600125, selective JNK inhibitor, inhibited H₂O₂-induced PC12 cell death. In this study, to confirm the role of p38 MAPK in H₂O₂-induced apoptosis, we examined the effect of SB203580, selective inhibitor of p38 MAPK, on PC12 cells. PC12 cells were pretreated with SB203580 (10 and 20 μM) for 30 min, and then further treated with 200 μM H₂O₂ for 24 h. The viability of cells incubated...
with 200 μM H₂O₂ for 24 h was 42.6 ± 5.7% of the control value, and this increased to 64.5 ± 7.1% and 73.1 ± 4.2% when they were pretreated with SB203580 at 10 and 20 μM, respectively (Fig. 7A). The increased nuclear condensation of the cells exposed to 200 μM H₂O₂ was significantly decreased by pretreatment with 20 μM SB203580 (Fig. 7B). The viability and nuclear condensation of the cells treated with 20 μM SB203580 only for 24 h did not exhibit significant alteration compared with control. These results indicate that p38 MAPK is involved in H₂O₂-induced apoptosis of PC12 cells.

4. Discussion

Polyphenols exhibit antiinflammatory, antiallergenic, anticarcinogenic, and antioxidant properties. Many studies have focused on the inhibition of risk factors associated with cancer and heart disease by polyphenols, but few studies have
investigated their role in brain functioning. Recently, dietary components that play important roles in neurodegenerative disease have received considerable attention, particularly those involving polyphenols. Cocoa is known to be abundant in polyphenols such as procyanidin B2. We previously reported the potential antitumor-promoting effects of cocoa polyphenols [15]. It has also been reported that epicatechin and catechin in cocoa have the potential to protect PC12 cells from cell death induced by Aβ [16]. However, cocoa commonly contains more procyanidins than epicatechin and catechin [27,28]. The present study demonstrated that CPF and procyanidin B2 potently increased the viability of PC12 cells treated with H2O2, indicating that they protect against H2O2-induced cytotoxicity.

Apoptosis is a gene-regulated process that involves changes in the expression of anti- and/or proapoptotic genes. Bcl-XL and Bcl-2 act as an antiapoptotic gene in parts of the endoplasmic reticulum and mitochondrial membrane, and thus has been shown to extend cell survival through blocking the apoptosis induced by cell death signals [5]. We confirmed that H2O2-induced Bcl-XL and Bcl-2 downregulation in PC12 cells, and that Bcl-XL and Bcl-2 were upregulated in PC12 cells treated with CPF or procyanidin B2. Furthermore, we clarified that apoptotic nuclear condensation and DNA fragmentation occurred in response to H2O2, which are considered biochemical hallmarks of apoptosis that have been observed in many types of cells undergoing apoptotic death. Both CPF and procyanidin B2 strongly mitigated these apoptotic phenomena.

Caspases are cysteine proteases that regulate apoptosis and mediate cell death. At least 14 mammalian caspase isoforms are known to exist as proenzymes that are cleaved and activated by apoptosis signals [29]. Caspase-3 plays an important role in apoptosis, chromatin condensation, and DNA fragmentation [30]. It was reported that the activation of caspase-3 by H2O2 treatment in vitro is a crucial effector of apoptosis [9]. In agreement with that study, the present results indicate that H2O2-induced caspase-3 cleavage in PC12 cells, and hence that H2O2-triggered apoptosis. CPF and procyanidin B2 rescued PC12 cells treated with H2O2 from caspase-3 cleavage. Loss of the mitochondrial membrane potential and activation of caspases leads to PARP cleavage that results in the cleaving of full-length PARP (116 kDa) to its 89-kDa form [31]. Our result clearly showed that CPF and procyanidin B2 attenuated cleavage of PARP in H2O2-treated PC12 cells.

There are multiple lines of evidence that the MAPK family of serine/threonine kinases plays a critical role in apoptosis signaling pathways. The major MAPKs have been verified as ERK, JNK, and p38. Phosphorylation of ERK plays a critical role as a cell survival factor against oxidative stress [32]. However, the roles of ERK in neuron death remain unclear [33]. On the other hand, it was reported that oxidative-stress-induced phosphorylation of JNK and p38 MAPK is required for neuron death and apoptosis [34,35]. A previous study showed that SP600125 and SB203580 – which are specific inhibitors of JNK and p38, respectively – prevent the PC12 cell death induced by H2O2 [26]. Other reports have also suggested that JNK and p38 MAPK are involved in H2O2-induced PC12 cell death [33,36]. In the present study, treatment with H2O2 resulted in phosphorylation of JNK and p38 MAPK within 15 min, and this was inhibited by CPF and procyanidin B2. The SB203580 treatment attenuated H2O2-induced cytotoxicity and nuclear condensation of PC12 cells. These results indicate that CPF and procyanidin B2 prevent the H2O2-induced apoptosis in PC12 cells by blocking the phosphorylation of JNK and p38 MAPK.

In summary, CPF and procyanidin B2 protect neurons from H2O2-induced apoptosis through inhibition of the phosphorylation of JNK and p38 MAPK. The protective effects of CPF and procyanidin B2 against apoptosis were confirmed by attenuation of nucleus condensation, DNA fragmentation, caspase-3 cleavage, PARP cleavage, and downregulation of Bcl-XL and Bcl-2. Therefore, CPF and procyanidin B2 might protect neurons against oxidative-stress-induced neurodegeneration.

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