Alzheimer's disease (AD) is a neurodegenerative disorder characterized by accumulation of amyloid β (Aβ)-containing plaques and neurofibrillary tangles in the brain [1], which are attributed to the cognitive impairment associated with AD [2]. Previous studies have demonstrated that AD is associated with oxidative stress resulting in protein oxidation, DNA oxidation, and lipid peroxidation [3,4]. 4-Hydroxynonenal (HNE), one of the aldehydic products of membrane lipid peroxidation, acts in AD as a downstream mediator propagating oxidative stresses induced by primary oxidant insults such as Aβ [5,6]. There are accumulating reports in the literature that the level of HNE is elevated in the brains of AD patients [7,8], especially localized in the Aβ deposition of AD brain tissues [9], which indicates that HNE contributes to the toxic effect of amyloid deposits leading to the development and progression of AD [1]. Because HNE-induced neuronal death is caspase-3 dependent [10], modulating the proteins involved in mitochondrial function might be a valuable target for the suppression of HNE-induced apoptosis. Various genes and their proteins are associated with progression of mitochondria-dependent apoptosis, and the Bcl-2 family comprises a group of apoptosis-regulating proteins. In this family, Bcl-2 and Bcl-XL are antiapoptotic genes associated with cell survival, and neuronal cells expressing Bcl-2 are reported to resist HNE-induced apoptosis owing to an increased level of glutathione [11].

Three major mitogen-activated protein kinases (MAPKs)—extracellular-signal-regulated protein kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAPK—are involved in early signaling mechanisms [12]. ERK is normally activated by growth factors and plays a key role in cell proliferation and differentiation, whereas JNK and p38 MAPK are activated by inflammatory cytokines and environmental stressors and act as essential mediators of apoptosis [12]. There are multiple lines of evidence that both JNK and p38 MAPK are involved in neuronal cell apoptosis induced by survival signal.
withdrawal or Aβ [11,13]. However, in the case of HNE-induced apoptosis of rat pheochromocytoma (PC12) cells, selective activation of the JNK pathway without the activation of ERK and p38 MAPK is necessary and sufficient for inducing apoptosis [14]. There is also accumulating evidence in the literature for the essential role of JNK in neuronal apoptosis. Inhibition of the JNK pathway suppresses the apoptosis induced by the withdrawal of nerve growth factor in PC12 cells [15,16]. Taken together, these reports suggest that JNK represents a valuable therapeutic target for modulating neuronal apoptosis. JNKs are activated via activation of the MAPK kinases (MKKs) MKK4 and MKK7. In response to oxidative stress, MKK4 translocates to the cell body together with MKK7, where both MKKs activate JNK and promote apoptosis [17]. It is well established that the MKK4→JNK pathway plays a crucial role in neuronal apoptosis [18–20]. Introducing dominant-negative forms of MKK4, JNK, or c-Jun (a JNK substrate) blocks the cell death induced by trophic factor deprivation in PC12 cells and sympathetic ganglia [16,21,22].

Antioxidant mechanisms in neurons might prevent apoptosis mediated by reactive oxygen species (ROS) [23,24]. Cocoa exhibits higher antioxidant activity than red wine, green tea, and black tea [25] and exerts beneficial effects on cardiovascular diseases [26,27], some types of cancers [28], and Aβ-induced neurotoxicity [29]. A recent clinical study showed the potential benefits of the consumption of flavanol-rich cocoa on cognitive tasks and brain perfusion [30,31]. Procyanidin B2 [epicatechin-(4→8)-epicatechin] (Fig. 1), a major polyphenolic compound present in cocoa, is widespread in nature and in processed foodstuffs such as cocoa, chocolate, red wine, and fruit juice [32]. The results of epidemiological research suggest that procyanidins on neuronal apoptosis and AD are not fully understood.

To investigate the potential neuroprotective effects of the cocoa procyanidin fraction (CPF) and procyanidin B2 and their mechanism, this study determined whether CPF and procyanidin B2 protect PC12 cells from apoptosis induced by HNE. We confirmed that HNE-induced apoptosis was mediated by ROS accumulation, MKK4–JNK activation, poly(ADP-ribose) polymerase (PARP) cleavage, Bcl down-regulation, and caspase-3 activation in PC12 cells. We also found that cocoa procyanidins protected neuronal cells from HNE-induced apoptosis by blocking both ROS accumulation and MKK4 activity.

Materials and methods

Sample preparation

Cocoa procyanidins were extracted from commercially available cocoa powder as described previously [28]. 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 cocoa extract, which was loaded onto a styrene-based adsorption resin column (60×450 mm; HP-20, Mitsubishi, Japan), washed with 20% (v/v) aqueous ethanol, and then eluted with 60% (v/v) aqueous ethanol. The eluted CPF was concentrated at 50 °C under reduced pressure and then frozen and dried.

Chemicals

Procyanidin B2 was purchased from Funakoshi (Funakoshi, Japan). HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Trypan blue solution (0.4%), 4,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) solution, 2,7’-dichlorofluorescin diacetate (DCFH-DA) and (−)-epicatechin 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-caspase-3, anti-JNK, and anti–MKK4 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-phosphorylated-JNK, and anti-phosphorylated–MKK4 antibodies were purchased from Cell Signaling (Beverly, MA, USA). The MKK4 assay kit was obtained from Upstate Biotechnology (Lake Placid, NY, USA). CNBr-Sepharose 4B and [γ−32P]ATP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). SP600125 was obtained from Biosciences (Ellisville, MO, USA). All other chemicals used were of analytical grade.

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.

MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay provides a sensitive measurement of the normal metabolic status of cells, particularly that of mitochondria, which reflects early cellular redox changes. PC12 cells (2×104 cells/well in 96-well plates) were incubated at 37 °C with 20 μM HNE for 24 h with or without pretreatment with CPF or procyanidin B2 and then treated with the MTT solution (final concentration, 1 mg/ml) for 2 h. The formazan crystals formed in intact cells were dissolved in dimethyl sulfoxide, 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.

Trypan blue exclusion assay

The trypan blue exclusion assay is based on trypan blue dye interacting with a cell if its membrane is damaged, because the chromophore is excluded only from viable cells. PC12 cells (105 cells/well in six-well plates) were suspended after being incubated at 37 °C with 20 μM HNE 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 phosphate-buffered saline (PBS). The total cell suspension was mixed with 200 μl of 0.4% trypan blue staining.

Fig. 1. Chemical structure of procyanidin B2.
solution for 5 min at room temperature. The cells were then loaded into a hemocytometer, and those exhibiting dye uptake were counted under a microscope. The percentage of stained cells was counted by scoring 150 cells.

**DAPI staining assay**

The fluorescent dye DAPI was used to detect the nuclear fragmentation that is a characteristic of apoptotic cells. PC12 cells (5 × 10⁴ cells/well in 24-well plates) were incubated at 37 °C with 20 μM HNE for 24 h with or without pretreatment with CPF or procyanidin B2 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). After 10 min of incubation, the cells were washed with PBS, and the plates were observed under a fluorescence microscope (Olympus Optical, Japan). The degree of nuclear fragmentation was evaluated by counting the percentage of DAPI-stained cells in 100–120 randomly selected cells.

**Flow cytometry using a fluorescein-activated cell sorter**

A flow cytometry method was used to assess the percentage of apoptotic cells. PC12 cells (1.6 × 10⁶ cells/8 ml in an 8.5-cm dish) were incubated at 37 °C with 20 μM HNE for 24 h with or without treatment with CPF or procyanidin B2. After treatment, PC12 cells were dissociated using trypsin and centrifuged at 200 g for 10 min. The pellets were resuspended in ice-cold 70% (v/v) ethanol and fixed overnight at 4 °C. The cells were then centrifuged at 200 g for 10 min and resuspended in 800 μl of PBS containing 16 μl of PI solution and 20 μg/ml RNase A at room temperature for 30 min in the dark. Cell fluorescence was measured with a flow cytometer (FACSCalibur, Becton–Dickinson, San Jose, CA, USA). In flow-cytometry histograms, apoptotic cells exhibit DNA fluorescence in the subdiploid region, which is well separated from the normal G1 peak. The percentage of apoptotic cells that accumulated in the sub-G1 phase was analyzed with CellQuest software (version 3.1f, Becton–Dickinson). Ten thousand cells in each sample were analyzed.

**DCFH-DA assay**

We measured the accumulation of intracellular ROS using the fluorescent probe DCFH-DA. DCFH-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals (mainly H₂O₂) to be converted into its fluorescent product, DCF, which is retained within the cells and thus provides an index of oxidation in the cell cytosol. After the cells were cultured in a 24-well plate for 24 h, 20 μg/ml DCFH-DA was loaded for 20 min. Cells were preincubated for 30 min with CPF (5 and 10 μg/ml) or procyanidin B2 (10 and 20 μM) and then exposed to 20 μM HNE for 5 min. Cells were examined at
535 nm with a fluorescence spectrophotometer (Infinite M200; Tecan Trading, Switzerland), with excitation at 485 nm.

Western blot analysis

PC12 cells (2 \times 10^5 cells/4 ml in a 6-cm dish) were incubated at 37 °C with 20 μM HNE 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 fluoride 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. The 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 a ratio of 1:1 and then exposing them to photographic film for a few minutes.

Direct MKK4 kinase assays

Direct kinase assays were performed in accordance with the instructions provided by Upstate Biotechnology. In brief, every reaction contained 20 μl of assay dilution buffer [Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.1 mM Na2VO4, 0.1% 2-mercaptoethanol, 1 mg/ml bovine serum albumin (BSA)], a magnesium–ATP-cocktail buffer, and 2 μg of unactivated JNK1α1. A 10-μl aliquot was removed after the reaction mixture was incubated at 30 °C for 30 min, to which 25 μg of ATF-2 and 10 μl of diluted [γ-32P]ATP solution were added. A 10-μl aliquot was then removed after this reaction mixture was incubated at 30 °C for 15 min, to which 10 μl of diluted [γ-32P]ATP solution was added. This mixture was incubated for 15 min at 30 °C, and then 20-μl aliquots were transferred onto p81 paper and washed three times with 0.75% phosphoric acid for 5 min per wash and once with acetone for 2 min. The radioactive incorporation was determined using a scintillation counter. Each experiment was performed three times.

Fig. 4. Effects of CPF and procyanidin B2 on HNE-induced apoptosis in PC12 cells. Apoptotic cells were analyzed by flow cytometry after the addition of PI solution for staining, and the percentage of apoptotic cells was determined from their accumulation in the sub-G1 phase. Cells were preincubated for 30 min with CPF or procyanidin B2 and then exposed to 20 μM HNE for 24 h: (A) no treatment, (B) 20 μM HNE, (C) 20 μM HNE + 5 μg/ml CPF, (D) 20 μM HNE + 10 μg/ml CPF, (E) 20 μM HNE + 10 μM procyanidin B2, and (F) 20 μM HNE + 20 μM procyanidin B2.
Direct pull-down assays

Recombinant MKK4 (0.2 μg) was incubated with the CPF-Sepharose 4B or procyanidin B2-Sepharose 4B (or Sepharose 4B as control) beads (100 μl, 50% slurry) in reaction buffer [50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 μg/ml BSA, 0.02 mM PMSF, and 1× protease inhibitor mixture]. After incubation with gentle rocking overnight at 4 °C, the beads were washed five times with buffer [50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF], and proteins bound to the beads were analyzed by immunoblotting.

Statistical analysis

When necessary, data are expressed as means ± SD 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.

Results

CPF and procyanidin B2 inhibit HNE-induced PC12 cell death

We first examined the possible protective effects of CPF and procyanidin B2 against HNE-induced PC12 cell death using the MTT reduction assay. The HNE-induced reduction in PC12 cell viability was reversed by treatment with CPF and procyanidin B2. We found that the percentage of viable cells when cells were incubated with 20 μM HNE alone for 24 h was 51.6 ± 9.6% of the control value, and this was increased to 71.6 ± 3.6, 85.4 ± 3.2, 71.9 ± 5.7, and 87.2 ± 4.0% in cells pretreated with CPF at 5 and 10 μg/ml or procyanidin B2 at 10 and 20 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 percentage of viable cells after exposure to 20 μM HNE for 24 h was 64.9 ± 8.6%, and this was increased to 82.2 ± 5.8, 91.6 ± 3.5, 86.0 ± 4.5, and 89.9 ± 4.2% in cells pretreated with CPF at 5 and 10 μg/ml or procyanidin B2 at 10 and 20 μM, respectively (Fig. 2B). Thus, the HNE-induced reduction in PC12 cell viability was protected by CPF and procyanidin B2 in a dose-dependent manner.

CPF and procyanidin B2 attenuate HNE-induced apoptosis of PC12 cells

Apoptosis is morphologically characterized by nuclear condensation, and cells in the sub-G1 phase are regarded as apoptotic. Two indices were applied to identify whether HNE induced cell death via apoptosis: (1) nuclear condensation, as measured by fluorescence microscopy using DAPI dye, and (2) apoptotic cell count, as measured by flow cytometry using PI dye. PC12 cells were preincubated for 30 min with CPF (5 and 10 μg/ml) or procyanidin B2 (10 and 20 μM) and then exposed to 20 μM HNE for 24 h. DAPI staining data indicated that HNE induced nuclear condensation in a large proportion of the PC12 cells, and this was significantly decreased by treatment with CPF or procyanidin B2 (Fig. 3A and B). Staining with PI dye indicated that 82.2 ± 3.6, 85.4 ± 3.2, 71.9 ± 5.7, and 87.2 ± 4.0% in cells pretreated with CPF at 5 and 10 μg/ml or procyanidin B2 (10 and 20 μM) for 30 min before exposure to 20 μM HNE for 24 h reduced the percentage of apoptotic cells to 19.03, 5.31, 16.11, and 3.45%, respectively (Fig. 4). These results indicated that CPF and procyanidin B2 protected against HNE-induced apoptosis of PC12 cells.

CPF and procyanidin B2 prevent HNE-induced intracellular ROS accumulation, cleavage of PARP, down-regulation of Bcl-XL and Bcl-2, and activation of caspase-3

To confirm whether the protection offered by CPF and procyanidin B2 is an antioxidative effect, we examined the intracellular ROS accumulation using DCFH–DA analysis. HNE induced intracellular ROS accumulation, and it was prevented by pretreatment with CPF and procyanidin B2 (Fig. 5A). Because PARP cleavage represents a biochemical hallmark of apoptosis, we also examined PARP cleavage to elucidate the molecular mechanism underlying the protective effects of CPF and procyanidin B2 against HNE-induced cell death. We measured the intracellular concentrations of pro-PARP (116 kDa) and cleaved PARP (89 kDa). Pretreatment with CPF (5 and 10 μg/ml) or procyanidin B2 (10 and 20 μM) for 30 min before exposure to 20 μM HNE for 24 h inhibited HNE-induced cleavage of PARP in a dose-dependent manner (Fig. 5B). Because oxidative stress-induced apoptosis is closely related to mitochondrial dysfunction [36], we investigated whether HNE-induced mitochondrial dysfunction is mediated by the down-regulation of Bcl-2 and Bcl-XL. CPF (5 and...
10 μg/ml) and procyanidin B2 (10 and 20 μM) inhibited HNE-induced down-regulation of Bcl-2 and Bcl-XL in a dose-dependent manner (Fig. 5B). Loss of the mitochondrial membrane potential activates caspases that are cleaved and activated during apoptosis from their initial proenzyme forms. Among many classes of caspases, caspase-3 has been shown to be an important regulator of apoptosis. Therefore, we assessed the presence of procaspase-3 at 32 kDa and the cleaved caspase-3 at 19 kDa. Cleavage of caspase-3 was evident after 24 h of exposure to HNE, and this was markedly reduced by treatment with CPF (5 and 10 μg/ml) or procyanidin B2 (10 and 20 μM) (Fig. 5C).

CPF and procyanidin B2 block HNE-induced JNK phosphorylation by directly modulating MKK4 activity

Because MKK4 (an upstream kinase of JNK) and JNK are reportedly involved in HNE-induced apoptosis of PC12 cells [14,37], we investigated whether the suppression of apoptosis by CPF and procyanidin B2 is mediated by the regulation of the MKK4–JNK pathway. Western blot analysis revealed that HNE-induced phosphorylation of JNK and MKK4 was inhibited by pretreatment with CPF and procyanidin B2 (Fig. 6A). To elucidate whether CPF and procyanidin B2 inhibited HNE-induced JNK phosphorylation by modulating MKK4 activity, we also examined the effects of CPF and procyanidin B2 on MKK4 activity. Direct kinase assays revealed that CPF and procyanidin B2 significantly inhibited MKK4 activity (Fig. 6B), and pull-down assays demonstrated that CPF and procyanidin B2 bound directly to MKK4 (Fig. 6D, lane 3). However, (−)-epicatechin did not inhibit MKK4 activity (Fig. 6C). These data suggest that the inhibitory effects of CPF and procyanidin B2 on HNE-induced JNK phosphorylation were in part caused by the direct inhibition of MKK4 activity.

To confirm the role of JNK in HNE-induced apoptosis, we examined the effect of SP600125, a selective inhibitor of JNK, on the apoptosis of PC12 cells. The inhibition of JNK resulted in the suppression of HNE-induced PC12 cell death (data not shown), and the HNE-induced sub-G1 arrest of cells was significantly decreased by pretreatment with 20 μM SP600125 (Fig. 7A). Staining with PI had indicated that 31.13% of cells incubated with 20 μM HNE for 24 h died via apoptosis, and treatment with SP600125 significantly decreased this to 13.29%. Treatment with 20 μM SP600125 alone for 24 h had no significant effects on the viability and sub-G1 arrest of cells compared to the untreated group (data not shown). SP600125 also inhibited PARP

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**Fig. 6.** JNK activation is down-regulated by CPF and procyanidin B2 by directly inhibiting MKK4 activity. (A) CPF and procyanidin B2 inhibit HNE-induced phosphorylation of JNK and MKK4 in PC12 cells. Cells were preincubated for 30 min with CPF (5 and 10 μg/ml) or procyanidin B2 (10 and 20 μM) and then exposed to 20 μM HNE for 5 min. The protein levels of phosphorylated JNK and MKK4 were measured by Western blot analysis. Total JNK and MKK4 were measured to ensure equal protein loading. (B, C) CPF and procyanidin B2, but not (−)-epicatechin, significantly suppress MKK4 activity. Direct MKK4 assays were performed as described under Materials and methods. Data are mean and SD values for three independent experiments. ###p<0.001 compared with control. **⁎⁎⁎p<0.001 compared with active MKK4. (D) CPF and procyanidin B2 bind with MKK4. The direct MKK4–CPF and MKK4–procyanidin B2 binding was confirmed by immunoblotting using an antibody to MKK4: lane 1 (input control), MKK4 protein standard; lane 2 (control), Sepharose 4B used to pull down MKK4; and lane 3, MKK4 pulled down using CPF–Sepharose 4B or procyanidin B2–Sepharose 4B affinity beads.
Western blot analysis.

HNE in PC12 cells. Cells were preincubated for 30 min with SP600125 and then exposed to residues of proteins to impair their function [38]. HNE affects various electrophilic species that can form covalent adducts with amino acid residues of proteins to impair their function [38].

Discussion

HNE released from the peroxidation of membrane lipids is an electrophilic species that can form covalent adducts with amino acid residues of proteins to impair their function [38]. HNE affects various biological processes including proliferation, differentiation, and apoptosis by modulating the expression of genes that regulate the cell cycle, such as p53, and other signaling proteins such as MAPK [39]. HNE seems to be unique among aldehydes in being able to trigger apoptosis by lipid peroxidation, because other aldehydic products do not cause apoptosis in PC12 cells and hippocampal neurons [40].

Accumulating data suggest that free radicals generated from Aβ peptide induce injury and cell death of neurons in AD [41] and that Aβ peptide increases the free and protein-bound forms of HNE [40].

Previous study revealed that the increased levels of proteins modified by HNE were detected in H2O2-treated hippocampal neurons, indicating that HNE can be a downstream mediator of H2O2-induced apoptosis signaling [42]. Another study also demonstrated that the H2O2-induced apoptosis, at least in part, is transduced via 4-HNE [43]. However, this does not necessarily mean that the apoptotic pathways evoked by H2O2 and 4-HNE are completely overlapped in AD [44]. H2O2-induced apoptosis of HT22 mouse hippocampal cells does not involve HNE production, indicating that mechanisms of H2O2-induced apoptosis are different from those induced by HNE [45]. Also, either HNE or H2O2 alone did not evoke the same amount of apoptosis caused by Aβ in SK-N-BE neuroblastoma cells, indicating that both HNE and H2O2 generation is required to reproduce the complete activation of Aβ-induced neuronal death [13]. Furthermore, during signaling, H2O2 and HNE target different proteins and induce different modifications of proteins [44]. Lipid peroxidation is not the only result caused by H2O2; protein oxidation, DNA strand breakage, and base modification are all major events evoked by both H2O2 and further production of highly reactive hydroxyl radicals [46]. Furthermore, HNE induces oxidative modifications of tau and promotes its aggregation, resulting in the formation of neurofibrillary tangles [47], and also covalently modifies Aβ, triggering its aggregation [48]. Therefore, to have an applicable effect against neuronal disease like AD, it is important for neuroprotective agents to show inhibitory effects on HNE-induced neuronal apoptosis specifically evoked by HNE. However, further studies are necessary to clarify whether HNE signaling is integrated with the signaling caused by other oxidative stresses including H2O2.

Cocoa products are sources of abundant polyphenols such as catechin, epicatechin, and procyanidin oligomers that comprise catechin and epicatechin subunits, and their antioxidant capacity is higher than that of other polyphenol-rich foods and beverages such as apples, red wine, and brewed black tea [27]. Cocoa polyphenols exert beneficial effects on cardiovascular diseases by inhibiting low-density lipoprotein oxidation [33] and modulating platelet activation [26], and they also affect immune responses [49]. Procyanidin B2 is one of the major compounds of cocoa, and the content of this compound in cocoa is higher than that of other procyanidin oligomers [50,51]. However, the effects of cocoa procyanidins on oxidative neuronal cell death and the underlying protective mechanisms have not been reported, whereas those of epicatechin and catechin have been reported previously [29]. The present study revealed that cocoa procyanidins attenuate HNE-induced PC12 cell death.

Apoptotic cells are characterized by cell shrinkage, chromatin condensation, DNA fragmentation, and an increased sub-G1 fraction. We investigated nuclear condensation and the rate of apoptosis to determine whether the protective effects of CPF and procyanidin B2 against HNE-induced PC12 cell death are mediated by their anti-apoptotic activities. HNE activates nucleus condensation and DNA fragmentation in PC12 cells [11], and in this study CPF and procyanidin B2 attenuated the induction of nuclear condensation. The up-regulation of proteins that regulate the cell cycle, such as p53 and p21, is involved in cell-cycle arrest and subsequent apoptosis, and cycloheximide, an inhibitor of protein synthesis, inhibits cell-cycle...
arrest and apoptosis by modulating p53 and p21 levels [52]. Cycloheximide prevents HNE-induced apoptosis in PC12 cells [11], which implies that cell-cycle regulation contributes to HNE-induced PC12 cell apoptosis. We therefore quantified the apoptosis rate as the percentage of cells in the sub-G1 phase and found that CPF and procyanidin B2 can inhibit the increased apoptosis induced by HNE.

HNE modulates multiple mechanisms that occur during apoptosis, such as increased ROS levels and disruption of mitochondrial function. We showed that CPF and procyanidin B2 suppressed the ROS accumulation induced by HNE. Inhibition of Bcl-2 expression causes mitochondrial dysfunction by releasing cytochrome c from the mitochondria, which leads to the subsequent activation of caspase cascades [36,53]. PC12 cells that overexpress Bcl-2 are resistant to apoptosis induced by HNE or oxidative stress [11]. Reduction of the mitochondrial membrane potential and activation of caspases subsequently modulate cleavage of PARP from its full-length form (116 kDa) to the cleaved form (89 kDa). Unlike Aβ-induced apoptosis, HNE-induced PC12 cell apoptosis is attributable to the activation of a caspase-3-dependent pathway [10,54]. This study clearly showed that CPF and procyanidin B2 inhibited HNE-induced caspase-3 cleavage, PARP cleavage, and Bcl down-regulation, and this inhibition might lead to the suppression of PC12 cell apoptosis.

Members of the MAPK family are involved in early signaling mechanisms in response to various stimuli [14], and previous studies have shown the differential activation of MAPKs induced by HNE. Whereas JNK and p38 MAPK are activated by HNE in rat hepatic epithelial cells [55], exposure to HNE results in the activation of ERK, JNK, and p38 MAPK in vascular endothelial cells [36]. In PC12 cells, HNE selectively activates JNK during apoptosis via the ASK1–MKK4–JNK pathway, and JNK activation as an early response to HNE is sufficient to induce apoptosis because it subsequently activates downstream effectors including activator protein-1 [37]. We determined that CPF and procyanidin B2 inhibited this early activation of JNK, which contributes to the suppression of apoptosis. This study also found that pretreatment with a specific inhibitor of JNK (SP600125) attenuated the HNE-induced apoptosis by blocking caspase-3 activation, which is consistent with previous research demonstrating the critical role of JNK in HNE-induced apoptotic signaling in PC12 cells. Furthermore, our results indicate that the involvement of JNK in apoptosis is mediated by the activation of caspase-3 as a downstream target of JNK. JNKs can be activated by MKks including MKK4 and MKK7, and MKks can be activated by several MKks including MEKK1, MEKK2, MEKK3, MEKK4, the mixed-lineage protein kinase group (MLK1, MLK2, MLK3, and DLK), the apoptosis-signal-regulating kinase group (ASK1 and ASK2), TAK1, and TPL2 [57,58]. Activation of the ASK1–MKK4–JNK cascade has been found in models of neuronal degeneration [59]. It is also reported that Aβ targets the ASK1–JNK proapoptotic pathway through ROS production in PC12 cells [60]. We have previously reported that CPF and procyanidin B2 inhibited H2O2-induced JNK phosphorylation in PC12 cells, but upstream kinases regulating H2O2-induced JNK phosphorylation have not been elucidated [61]. In the present study, CPF and procyanidin B2 attenuated HNE-induced apoptosis by blocking JNK phosphorylation, and this was caused by inhibiting the activity of MKK4, an upstream regulator of JNK. However, (–)-epicatechin, a monomer polyphenol, had no effect on MKK4 activity, indicating that the oligomeric procyanidins may be more effective at inhibiting MKK4 activity than epicatechin monomer. Furthermore, CPF and procyanidin B2 also inhibited MKK4 phosphorylation in addition to MKK4 activity, which suggests that CPF and procyanidin B2 can regulate the upstream regulators of MKK4 as well as MKK4 activity itself. This study revealed that MKK4–JNK phosphorylation was influenced by ROS accumulation, from which it can be suggested that the inhibition effects of the MKK4–JNK pathway are due to antioxidative effects of CPF and procyanidin B2.

In conclusion, we have confirmed that HNE-induced apoptotic signaling mechanisms are related to JNK activation and caspase-3 activation in PC12 cells. CPF and procyanidin B2 inhibited HNE-induced apoptosis characterized by the induction of nuclear condensation and DNA contents in the sub-G1 phase. These antiapoptotic effects of CPF and procyanidin B2 were mediated by blocking the activation of the MKK4–JNK pathway, as well as ROS accumulation, and the subsequent suppression of caspase-3 cleavage, PARP cleavage, and down-regulation of Bcl proteins. Together these results indicate that the suppression of MKK4 activity by CPF and procyanidin B2 contributes to antiapoptotic effects in PC12 cells by inhibiting JNK phosphorylation, which makes MKK4 a possible molecular target of CPF and procyanidin B2 for suppressing neuronal apoptosis.

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