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Original Contribution

Cocoa procyanidins attenuate 4-hydroxynonenal-induced apoptosis of PC12 cells by directly inhibiting mitogen-activated protein kinase kinase 4 activity

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ABSTRACT

Neurodegenerative disorders such as Alzheimer's disease (AD) are associated with oxidative stress, and it has been suggested that apoptosis is a crucial pathway in neuronal cell death in AD patients. 4-Hydroxynonenal (HNE), one of the aldehydic products of membrane lipid peroxidation, is reported to be elevated in the brains of AD patients and mediates the induction of neuronal apoptosis in the presence of oxidative stress. In this study, we investigated the HNE-induced apoptosis mechanism and the protective effects of the cocoa procyanidin fraction (CPF) and its major antioxidant procyanidin B2 against the apoptosis induced by HNE in rat pheochromocytoma (PC12) cells. HNE-induced nuclear condensation and increased sub-G1 fraction, both of which are markers of apoptotic cell death, were inhibited by CPF and procyanidin B2. Intracellular reactive oxygen species (ROS) accumulation was attenuated by pretreatment with CPF and procyanidin B2. CPF and procyanidin B2 also prevented HNE-induced poly(ADP-ribose) polymerase cleavage, antiapoptotic protein (Bcl-2 and Bcl-X_L) down-regulation, and caspase-3 activation. Activation of c-Jun N-terminal protein kinase (JNK) and mitogen-activated protein kinase kinase 4 (MKK4) was attenuated by CPF and procyanidin B2. Moreover, CPF and procyanidin B2 bound directly to MKK4 and inhibited its activity. Data obtained with SP600125, a selective inhibitor of JNK, revealed that JNK is involved in HNE-induced apoptosis through the inhibition of PARP cleavage and caspase-3 activation in PC12 cells. Collectively, these results indicate that CPF and procyanidin B2 protect PC12 cells against HNE-induced apoptosis by blocking MKK4 activity as well as ROS accumulation.

Alzheimer 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-X_L 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

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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-(4β-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 procyanidin B2 can exert several physiological effects, such as antioxidant activity [33], antitumor effects [34], and protection against DNA damage induced by Fe(II)/H_2O_2 [35]. Cocoa and its polyphenol components epicatechin and catechin individually can suppress Aβ-induced PC12 cell apoptosis, and a mixture of epicatechin and catechin exerts synergistic effects [29]. Although procyanidins comprise more than two types of catechins and are more abundant in cocoa than either epicatechin or catechin, the protective effects of procyanidins on neuronal apoptosis and AD are not fully understood.

To investigate the potent 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.

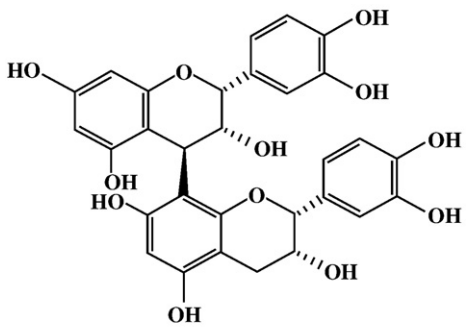

Fig. 1. Chemical structure of procyanidin B2.

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 f × 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-X_L, 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-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). SP600125 was obtained from Bioscience (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% CO_2 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 (2×10^4 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 dark blue 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 (10^5 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