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Epicatechin and Catechin in Cocoa Inhibit Amyloid β Protein Induced Apoptosis

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To elucidate additional health benefits of cocoa phytochemicals on the neurotoxicity induced by amyloid β protein (Aβ), PC12 cells were treated with toxic peptide ($A\beta_{25-35}$) and the effects of epicatechin, catechin, and cocoa were studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, lactate dehydrogenase (LDH) release, and trypan blue exclusion methods. Significant increase in neuronal cell death was observed on PC12 cells treated with $A\beta_{25-35}$ (25 μM), while epicatechin and catechin and their mixture prevented the Aβ-induced neuronal cell death. Aβ treatment also led to the increased membrane instability of PC12 cells. The membrane protective effects of the phenolics determined by LDH release and trypan blue exclusion assays demonstrated that epicatechin, catechin, and their mixture protect cellular membrane from Aβ-induced cytotoxicity. In these three different cell viability assays, the mixture of epicatechin and catechin showed the highest protective effect and synergistic activity. The present results showed that the major flavonoids of cocoa, epicatechin and catechin, protect PC12 cells from Aβ-induced neurotoxicity, and suggest that cocoa may have anti-neurodegenerative effect in addition to other known chemopreventive effects.

KEYWORDS: Alzheimer's disease; amyloid β protein; catechin; cocoa; epicatechin

INTRODUCTION

Alzheimer's disease (AD) is one of the major neurodegenerative disorders for which no treatment is available. Amyloid β protein (Aβ), in the form of insoluble fibril deposits, is the important constituent of senile plaques in AD patients, and it has been proposed to be the cause of the neurodegeneration that occurs in AD brains (*1*). $A\beta_{1-42}$, $A\beta_{1-40}$, and $A\beta_{25-35}$ are directly toxic to neuronal cell cultures at high micromolar concentrations. The observed cell death has been correlated with an effect of amyloid peptides on the membrane integrity as determined by lipid peroxidation. Furthermore, it has been shown that low nanomolar concentrations of Aβ peptides increase the susceptibility of the plasma membrane to additional insults (*2*).

Oxidative stress in AD may result from aging, energy deficiency, inflammation, or excessive production of Aβ. The link between Aβ and oxidative stress in AD brain is not clear, due to the fact that studies of postmortem tissue cannot reveal whether these amyloid deposits are the byproducts of neurodegeneration or precede the degenerative process. However, it has been suggested that Aβ toxicity is associated with increases in reactive oxygen species (ROS), including the peroxide H_2O_2 (*1*, *3*). Overproduction or impaired clearance of Aβ is believed by many researchers to be a critical step in the development of AD. Interestingly, oxidative stress also induces the expression and misprocessing of amyloid precursor protein, leading to the generation of amyloidogenic fragments (*4*). This process can result in a potentially debased cycle whereby oxidative stress leads to Aβ, and Aβ leads to more oxidative stress, neuronal dysfunction, and ultimately neuronal death.

Recently, attention has focused on the potential protective effects of phytochemicals against the neuronal deficits associated with neurodegenerative diseases. The protection of fruits and vegetables against chronic disease such as AD has been ascribed to their various antioxidants (*5*). Flavan-3-ols such as epicatechin (EC) and catechin (CE) are polyphenolic phytochemicals, commonly present in cocoa, green tea, red wine, and various fruit (*6*). In our previous report, we showed that EC and CE have stronger antioxidant activity than vitamin C (*7*), and cocoa has more antioxidative phytochemicals and higher antioxidative activity than red wine, green tea, and black tea (*8*). Cocoa beans (*Theobroma cacao*) are very rich in polyphenols, in the form of procyanidin oligomers of epicatechin/catechin flavanols, comprising 12−48% of the dry weight of the whole bean (*9*). Spencer et al. (*10*) founded that cocoa-derived procyanidins are unstable under gastric acid condition and decompose essentially to EC or CE. Therefore, procyanidin oligomers cannot pass through small intestine, but their decomposition products, monomers, would be the major components for absorption via the small intestine (*11*). In the light of the high antioxidant properties of cocoa phenolics, cocoa water extract, EC, and CE are expected to show neuroprotective effects on dysfunction cells that are vulnerable to Aβ induced oxidative stress. Here, we have evaluated the neuroprotective effect of cocoa extract, EC, and CE on Aβ-induced neurotoxicity in cultured PC12 cells.

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MATERIALS AND METHODS

Materials. RPMI 1640 medium, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). Commercial unsweetened cocoa powder was obtained from a local market. All other chemicals were the products of Sigma (St. Louis, MO). Aβ_{25-35}, which is the most toxic peptide fragment derived from amyloid precursor protein, was dissolved in deionized distilled water at a concentration of 1 mM and stored at −20 °C. The stock solutions were diluted to the desired concentrations immediately before use. EC and CE (10−100 μmol/L) were added from stock solutions in deionized distilled water. The mixture of EC and CE was prepared by the same concentration of two phenolics (e.g. the 10 μM mixture of EC and CE is 10 μM EC + 10 μM CE). The hot water extracts of cocoa were added from stock solutions in deionized distilled water (1 mg of cocoa extract = 4.5 μg of CE and 18 μg of EC). A cup of cocoa includes about 34 mg of CE and 134 mg of EC, respectively (*12*). Controls were not pretreated with cocoa extracts, EC, CE, and Aβ. Cells were preincubated with cocoa extracts and the two phenolics for 10 min. After 10 min, 25 μM Aβ was treated for 24 h. In our experiments, PC12 cell viability was not changed by cocoa extract, EC, and CE (data not shown).

Cocoa Extraction. Cocoa powder (10 g) was dissolved in 50 mL of distilled water (ddH_2O) at 100 °C for 2 min. The cocoa extracts then were centrifuged in a DuPont Sorvall RC-5B refrigerated superspeed centrifuge Wilmington, DE) at 12 000*g* using a SLA 1500 rotor for 5 min, and the resulting supernatants were used as the final samples (*8*).

Cell Culture. Rat pheochromocytoma PC12 cells were propagated in RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, 50 units/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 5% CO_2.

MTT Reduction Assay. The method of Hertel et al. (*13*) was used with slight modifications in this experiment (*14*). PC12 cells were plated at a density of 10^4 cells/well on 96-well plates in 100 μL RPMI, and the cell viability was determined by the conventional MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. The cells were incubated with 0.5 mg of MTT/mL (final concentration) for 2 h at 37 °C, and the reaction was stopped by adding solution containing 50% dimethylformide and 20% sodium dodecyl sulfate (pH 4.8). The amount of MTT formazan product was determined by measuring absorbance using a micro-plate reader (Bio-Rad, Hercules, CA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Lactate Dehydrogenase (LDH) Release Assay. The LDH assay is a means of measuring either the number of cells via total cytoplasmic LDH or membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The LDH assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. If cell-free aliquots of medium from cultures given different treatments are assayed, then the amount of LDH activity can be used as an indicator of relative cell viability as well as a function of membrane integrity (*15*). Briefly, floating PC12 cells were spun down, 50 μL of the supernatants was transferred into new wells, and LDH was determined using the Sigma toxicology assay kit (St. Louis, MO). Damage to the plasma membrane was evaluated by measuring the amount of the intracellular enzyme LDH released into the medium.

Trypan Blue Exclusion Assay. The trypan blue exclusion assay was based on the capability of viable cells to exclude the dye. Since viable PC12 cells maintained membrane integrity, the cells did not allow trypan blue dye to pass through the cell membrane. Cells with damaged membrane appeared blue due to their accumulation of dye and were counted as dead. Five minutes after 0.4% trypan blue was added to PC12 cells, they were loaded into a hematocytometer and counted for the dye uptake. The number of viable cells was calculated as the percent of the total cell population (*14*).

Statistical Analysis. The results were expressed as means ± SD. Differences among different experimental groups were tested for significance using one-way analysis of variance (ANOVA; SPSS Inc., Chicago, IL), taking $P < 0.05$ as significant.

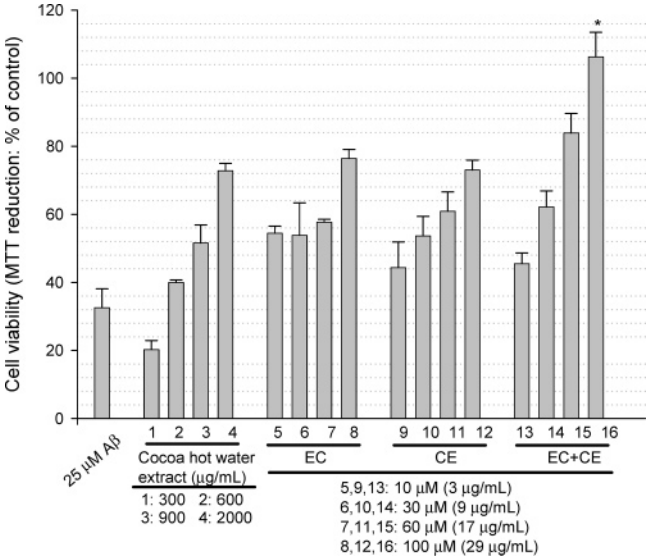

Figure 1. Cell-protective effect of cocoa extract, EC, and CE on Aβ-induced cytotoxicity in PC12 cell system. PC12 cells were pretreated for 10 min with various concentrations. After 10 min, the cells were treated with 25 μM Aβ for 24 h. Levels of cell viability were measured using the MTT assay as described in Materials and Methods. The viability of untreated control cells was defined as 100%. Results shown are means ± SD ($n = 3$). Data were compared with control group by one-way ANOVA test. Significant difference ($P < 0.05$) was observed on the Aβ-induced cytotoxicity; * was not significantly different from control.

RESULTS AND DISCUSSION

Oxidative stress is considered as a risk factor in the incidence and progression of cognitive declines that occur during normal cerebral aging and dementia and likely plays a critical role in various neurodegenerative processes, such as AD and Parkinson's disease (*16*, *17*). Recently, food-derived antioxidants such as vitamins and phytochemicals have received growing attention, because they are known to function as chemopreventive agents against oxidative damage. Many of the biological actions of phytochemicals including flavonoids have been attributed to their antioxidant properties (*18*).

Aβ associated with senile plaques formed in the brains of patients with AD was found to induce apoptosis in cultured neurons through the generation of H_2O_2 (*3*). Aβ_{25-35} has neurotoxicity and produces free radical adducts in aqueous solutions and sensitizes neurons to injury resulting from oxidative stress-induced neurotoxicity induced by glutamate or free radicals (*19*). In the present study, the Aβ cytotoxicity was evaluated by determining the percentage of MTT reduction after incubation of PC12 cells for 24 h with 25 μM Aβ_{25-35} (**Figure 1**). Aβ_{25-35} decreased the cell viability (about 72%), and its cytotoxic effects were gradually attenuated in the presence of cocoa extracts, EC, and CE. CE and cocoa extract increased cell viability concentration dependently. Especially, the treatment of a mixture of EC and CE dramatically increased the neuronal cell viability, and maximal protection was observed at 100 μM (105%). This synergistic effect of two phenolics significantly decreased Aβ-induced cytotoxicity. Therefore, this result clearly indicated that PC12 cell apoptosis by Aβ cytotoxicity was suppressed by pretreatment with the cocoa extracts, EC, and CE. In this respect, cocoa metabolites such as EC and CE may result in neuronal protection against Aβ-induced apoptosis after ingestion. In addition, MTT dye reduction assay is based on the catalytic activity of some metabolic enzymes in intact mitochondria (*13*). Therefore, these results suggest that

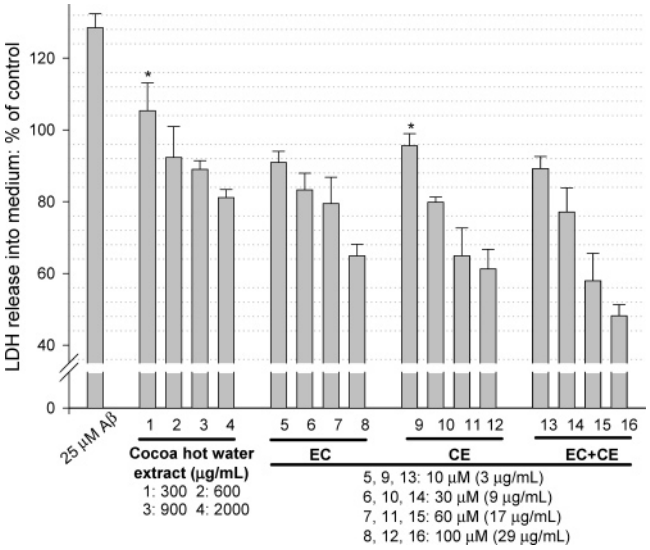

Figure 2. Effects of cocoa extract, EC, and CE on Aβ-induced membrane damage (LDH release into medium) in PC12 cells. PC12 cells were pretreated for 10 min with various concentrations. The cells were treated with Aβ (25 μM) for 24 h. LDH activity in culture supernatants was measured with a colorimetric LDH assay kit. All data are represented as the means ± SD ($n = 3$) and values obtained from three separate cultures. Statistical analysis indicated that the influence of the compounds used had significant effect on Aβ-induced membrane toxicity ($P < 0.05$); * was not significantly different from control.

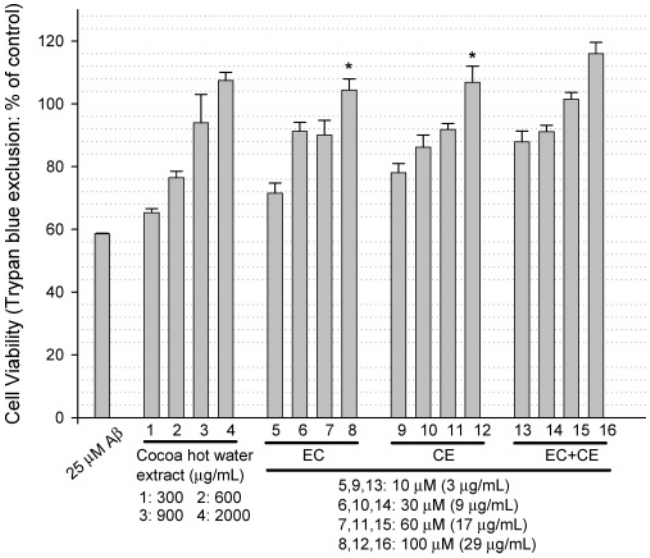

Figure 3. Inhibition of Aβ toxicity in PC12 cells after pretreatment with cocoa extract, EC, and CE as assessed by trypan blue exclusion staining followed by cell counting. PC12 cells were plated at low density in a 24-well plate. Cells were incubated with the phenolics for 10 min before the addition of 25 μM Aβ. Cultures were observed after an additional 24 h, and trypan blue exclusion staining was performed. Data are presented as mean ± SD for one representative triplicate determination and are expressed as the percent survival compared to the corresponding controls ($P < 0.05$); * was not significantly different from control.

PC12 cell protection by cocoa, EC, and CE is partially due to the mitochondrial protection mechanisms.

The mammalian brain is extraordinarily vulnerable to the cytotoxic effects of ROS (*20*). The superoxide anion and hydrogen peroxide formed by oxidative stress cannot be readily neutralized because of the low superoxide dismutase (SOD), catalase, and glutathione peroxidase activities present in the brain. Moreover, brain membrane lipids are very rich in polyunsaturated fatty acids, which are especially sensitive to free-radical-induced lipid peroxidation. The precise mechanism and the sequence of events by which free radicals interfere with cellular functions remain unclear, but lipid peroxidation is likely to be one of the most important events (*21*). To examine the membrane instability by Aβ-induced cytotoxicity, we have assessed the protective effect of cocoa extract, EC, and CE using the LDH release assay, measuring the activity of this stable enzyme released into the medium from apoptotic PC12 cells. A quantitative analysis of LDH activity can determine what percentage of cells is dead. Inhibition rates of cocoa extracts, EC, and CE against Aβ-induced membrane damage at different concentrations are shown in **Figure 2**. Treatment with Aβ caused an increase in LDH release into the medium, and a decrease in the number of viable cells (about 30%). Pretreatment with the cocoa extract, EC, and CE exerted efficient inhibitory activity on LDH release in PC12 cell system. EC and CE exhibited more effective protection than cocoa extract, and the mixture of EC and CE also showed a synergistic effect at high concentrations (≥ 60 μM). To confirm if cocoa extract, EC, and CE block the Aβ-induced membrane damage, the trypan blue exclusion assay that directly measures the viable cells maintaining the capability of excluding the dye and may reflect more precisely the integrity of viable cell membrane was also used. Aβ-induced cytotoxicity increased cell membrane damage and the cocoa extract, EC, and CE protected the PC12 cells from neurotoxicity (**Figure 3**). Our data also showed herein the protective effects of cocoa extract, EC, and CE like LDH release assay. Among these pretreatments, the mixture of EC and CE showed the highest protective effect at any concentrations. The maximal activity was 117% at 100 μM. Both results suggest that cocoa extract, EC, and CE protected the PC12 cells against Aβ-induced membrane damage through their antioxidant activity.

Cocoa phenolics, including EC and procyanidins, have potent antioxidant properties and lipid peroxidation activities in vitro (*22*, *23*). The extent of their antioxidative effects in vivo will be dependent on the absorption, metabolism, distribution, and excretion of these compounds within the body after oral ingestion. Recently, it has been reported that consumption of 80 g of semisweet chocolate that is rich in procyanidins resulted in 12-fold increases in plasma levels of EC after 2-h ingestion. In addition to the rise in EC levels, in the same time period there was also a significant increase in plasma antioxidant levels (31%) and a significant decrease (40%) in lipid peroxidation (*24*). In support of these findings, Richelle et al. (*25*) also observed an increase in plasma levels of EC after ingestion of a black chocolate (80 g) with levels peaking at 0.7 mmol/L between 2 and 3 h. Time-course analysis showed that the dominant metabolites in plasma and urine after EC consumption were conjugated forms of EC, such as a glucuronide and 3′-*O*-methylepicatechin (*26*). The ability of 3′-*O*-methylepicatechin and EC to protect against apoptotic cell death induced by hydrogen peroxide or oxidized low-density lipoprotein has been investigated, and both displayed the same degree of protection against apoptosis (*27*). In addition, the blood−brain barrier (BBB) formed by the endothelium of brain microvessels is a regulatory interface and selectively limits drug delivery to the central nervous system (CNS). Since the CNS controls cognitive functions such as learning and memory, penetration of active phytochemicals via the BBB is most important to allow them to exert their positive function. HPLC and LC−MS analysis showed the presence of EC glucuronide and 3′-*O*-methylated

epicatechin glucuronide in rat brain tissue after gavage (*28*). Therefore, it is possible that cocoa phytochemicals and their metabolites with beneficial antioxidant and bioavailabilities are able to penetrate the BBB and can protect against Aβ-induced cytotoxicity after oral consumption.

In conclusion, the protective effects of EC, CE, and cocoa against Aβ-induced apoptosis may be due to the dual effects of mitochondrial and membrane protection. Since cocoa is known to have strong antioxidant and chemopreventive activities, this additional benefit of anti-neurotoxicity suggests that daily consumption of cocoa may also provide an added health benefit by reducing the risk of neurodegenerative diseases such as AD.

ABBREVIATIONS USED

AD, Alzheimer's disease; Aβ, amyloid β; APP, amyloid precursor protein; BBB, blood−brain barrier; CE, catechin; CNS, central nervous system; ddH_2O, distilled water; EC, epicatechin; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase.

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