Research Article

Apple Procyanidins Suppress Amyloid β-Protein Aggregation

Toshihiko Toda,1,2 Tadahiro Sunagawa,3 Tomomasa Kanda,3 Motoyuki Tagashira,3 Takuji Shirasawa,1,4 and Takahiko Shimizu1

1 Molecular Gerontology, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan
2 JAC Co., Ltd., 1-2-7 Higashiyama, Meguro-ku, Tokyo 153-0043, Japan
3 Asahi Breweries, Ltd., 1-1-21 Midori, Moriya-shi, Ibaraki 302-0106, Japan
4 Ageing Control Medicine, Juntendo University Graduate School of Medicine, 3-1-3-10-201 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Correspondence should be addressed to Takahiko Shimizu, shimizut@tmig.or.jp

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Procyanidins (PCs) are major components of the apple polyphenols (APs). We previously reported that treatment with PC extended the mean lifespan of Caenorhabditis elegans (Sunagawa et al., 2011). In order to estimate the neuroprotective effects of PC, we investigated the antiaggregative activity of PC on amyloid β-protein (Aβ) aggregation, which is a pathological hallmark of Alzheimer’s disease. We herein report that PC significantly suppressed Aβ42 aggregation and dissociated Aβ42 aggregates in a dose-dependent manner, indicating that PC is a potent suppressor of Aβ aggregation. Furthermore, PC significantly inhibited Aβ42 neurotoxicity and stimulated proliferation in PC-12 cells. These results suggested that the PC and AP acted as neuroprotective factors against toxic Aβ aggregates.

1. Introduction

Polyphenols are comprised of several groups of compounds (e.g., anthocyanins, flavonols, and phenolic acids) and belong to a family of plant secondary metabolites that widely accumulate in plants as well as fruits [1]. The polyphenols extracted from apples (Malus pumila Mill., Rosaceae) mainly contain procyanidins (PCs), as well as known proanthocyanidins, leucocyanidins, and condensed tannins, which account for approximately 65% of apple polyphenols (AP) [2]. PC is formed by catechin oligomers composed of (−)-epicatechin and (+)-catechin monomers (MNs) [3]. PC is also found in a variety of fruits, berries, and several medicinal plants or plant components, such as grape (Vitis vinifera) seeds [4], bilberry (Vaccinium myrtillus) [5], hawthorn (Crataegus monogyna) [6], ginkgo (Ginkgo biloba) [7], tormentil (genus Potentilla) [8], and oak (genus Quercus) [9].

It has been reported that several polyphenols including PC show potential benefits to human health, such as antioxidant [10], antitumor [11], anti-inflammatory effects [12], and longevity [13], as well as protective effects on glucose consumption [14]. We have also revealed that apple PC showed anti-allergy [15], antitumor [16], and antiobesity effects [17] in a rodent model and longevity effects on Caenorhabditis elegans [2]. In this context, PC might be a promising polyphenol that can prevent age-related diseases.

Alzheimer’s disease (AD) is a typical age-related and progressive neurodegenerative disease with memory impairment in later life. AD is diagnosed by amyloid accumulation, which is observed as a deposition in the hippocampus and cerebral cortex, named a senile plaque, composed of amyloid β-protein (Aβ) [18]. Aβ42, which consists of 42-residues, is observed mainly in the core of senile plaques. The protein forms strong aggregates themselves that are associated with neurotoxicity in vitro [19]. Several polyphenols attenuate insoluble Aβ accumulation [20–22].

This study addressed the anti-neurodegenerative effects of PC and AP by investigating whether PC can prevent the aggregation of Aβ. The results demonstrated that apple
PCs suppress Aβ aggregation and cytotoxicity in vitro and strongly contribute to neuroprotection in AP.

2. Materials and Methods

2.1. Compounds. The APs were prepared from immature apples (Malus pumila Mill. cv. Fuji), and the methods employed for AP preparation were described previously [2]. The PCs (procyanidins, catechin oligomers) accounted for 63.8% of the AP, and the methods used to prepare the PC and monomer fractions were used as described previously [2]. The Aβ42 peptide was purchased from the Peptide Institute.

2.2. Thioflavin-T Fluorescence Assay. The aggregative abilities of Aβ42 were evaluated using the thioflavin-T (Th-T) method as described previously [22]. Various concentrations of test samples were coinubated with Aβ42 (final concentration 20 μM) for 6 to 48-hour at 37°C in 100 mM phosphate buffer (pH 7.4) containing 100 mM NaCl. In the case of posttreatment, Aβ42 (final concentration 20 μM) was preincubated without PC for 48 hours at 37°C, and then various concentrations of polyphenols were added and incubated for 0.5 to 5 hours at room temperature. The incubated samples were stored at −80°C until measurement. Th-T (final concentration 5 μM, Sigma-Aldrich) in 50 mM glycine-NaOH buffer (pH 8.5) was added to the samples and incubated for 30 minutes at room temperature. The measurements were performed on a SPECTRA max GEMINI XS fluorescence microplate reader (Molecular Devices). Fluorescence intensity was measured using 442 nm for excitation and 485 nm for emission. The percentage of Aβ aggregate inhibition was calculated by comparing the fluorescence values of the test samples with those of vehicle solutions with Aβ42.

2.3. Separation of Aβ Precipitation. Polyphenol samples (100 μg/mL AP, 65 μg/mL PC, and 35 μg/mL MN) were coinubated with Aβ42 (final concentration 20 μM) for 24 hours as described before [22]. A 300 μL aliquot of the reacted samples was centrifuged at 20,000 × g for 30 minutes at 4°C. The Aβ pellets were dried at room temperature before optical photographs were obtained using an SZX9 microscope (12.5-fold, OLYMPUS). The pellets were resolubilized in 6 M guanidine-HCl (pH 4.5–7.5, Sigma-Aldrich) and diluted by 15-fold RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS, Sigma-Aldrich). Aβ concentrations were measured using a DC protein assay kit (Bio-Rad Laboratories). The supernatants of the sedimented samples were denatured at 85°C for 2 minutes with EzApply solution (ATTO) containing 1% SDS and 50 mM dithiothreitol before separation by electrophoresis at 25 mA on 20% polyacrylamide gels in Tris-glycine buffer (SDS-PAGE). Soluble Aβ peptides in 5 μL of reacted solutions were detected using Coomassie brilliant blue staining (Quick-CBB, Wako Pure Chemical) according to the manufacturer’s protocol.

2.4. Estimation of Cell Viability. PC-12 cells (RCB0009, RIKEN BioResource Center) were used as a neural cell model in order to evaluate the cytotoxicity of Aβ peptides [23]. The experimental procedure was a previously described method [22]. Briefly, after the incubation of the PC-12 culture (2 × 10^4 cells per well) for 16 hours at 37°C, the cells were pretreated for 1 hour with or without various concentrations of filter-sterilized polyphenols, followed by treatment with 1 μM Aβ42 for an additional 36 hours. Then, cells were treated with thiazolyl blue tetrazolium bromide (MTT, final concentration 0.5 mg/mL, Sigma-Aldrich) for 4 hours at 37°C. After solubilization with SDS (Sigma-Aldrich), the rate of formazan formation was evaluated by measuring the absorbance at 570 nm using a VersaMax microplate reader (Molecular Devices). Data are given as percentages of the control values without PC and Aβ42.

2.5. Statistical Analyses. All data were presented as the means ± s.e.m. The StatMate III software package was used for all statistical analyses. Differences were analyzed by Student’s t-test, and multiple comparisons between groups were performed with Dunnett’s test for posthoc analysis. P values of <0.05 or <0.001 were considered to be statistically significant.

3. Results

3.1. In Vitro Anti-Aβ Aggregative Effects of Procyanidins. To examine the effect of PC on Aβ aggregation, we performed thioflavin-T (Th-T) fluorescence assays for Aβ42 (Figure 1). The PC comprised approximately 65% of the AP, and the remaining approximately 35% was regarded as the monomer fraction (MN) [2]. In the absence of PC, Aβ42 (20 μM) formed Th-T-binding aggregates 48 hours after incubation, whereas the Th-T fluorescence intensity was dramatically decreased in a dose-dependent manner by AP and PC (Figure 1(a)). In addition, 100 μg/mL AP and 32.5 μg/mL PC completely abrogated Aβ aggregation throughout the incubation period (Figure 1(a), ns; not significant compared with 0 hour, P < 0.01), while MN resulted in a limited decrease in the Aβ aggregation (Figure 1(a)). Since 32.5 μg/mL PC corresponded to 100 μg/mL AP, this result suggests that PCs have approximately 2-fold the anti-Aβ aggregative ability compared to AP. Interestingly, a bioavailable dose of PC (11.5 μg/mL), which had been detected in rat plasma after the oral administration of 1,000 mg AP/kg body weight [24], significantly suppressed Aβ aggregation (Figure 1(b)). Furthermore, low-dose AP and PC (1.0 and 0.65 μg/mL, resp.) significantly suppressed Aβ aggregation, while 0.35 μg/mL MN did not suppress the aggregation at any time during the incubation period (Figure 1(c)). These results suggest that apple PC effectively suppressed Aβ aggregation compared to MN, including epicatechins and catechins.

We next analyzed the inhibitory effect of PC on amyloid aggregation using a centrifugation method, to exclude the possibility that exogenous compounds affected Th-T fluorescence intensity [25]. We observed the typical aggregates of Aβ (20 μM) after 48 hours incubation (Figure 2(a); vehicle). Treatment with 100 μg/mL AP and 65 μg/mL PC completely diminished the aggregates, while 35 μg/mL MN treatment did not (Figure 2(a)). We also detected monomeric Aβ42 in the supernatant by SDS-PAGE following 100 μg/mL
Figure 1: Procyanidins suppress Aβ aggregation in vitro. (a) Dose-dependent suppression of Aβ42 aggregation by polyphenols as indicated by thioflavin-T (Th-T) analysis. Various concentrations of apple polyphenol (AP), procyanidins (PC), and monomers (MN) were incubated with Aβ42 (final concentration 20 μM) for 48 hours. (Differences from 0 hour (baseline), ns: not significant (P < 0.01)). (b, c) Time-dependent Aβ42 aggregation in the presence of polyphenols as indicated by the Th-T assay. (b) Aβ42 (20 μM) was incubated with AP (18.0 μg/mL), PC (11.5 μg/mL), and MN (6.5 μg/mL). (c) Aβ42 (20 μM) was incubated with AP (1.0 μg/mL), PC (0.65 μg/mL), and MN (0.35 μg/mL). (Differences compared to vehicle groups, *P < 0.001 (Dunnett’s test)). Values are the means ± s.e.m., n = 5.
Figure 2: Procyanidins extinguish Aβ precipitation. (a) Microscopic observation of Aβ42 precipitates by centrifugation in the presence of polyphenols. The aggregates were observed by 12.5-fold magnification. (b) SDS-PAGE analysis of soluble Aβ42 peptide of supernatants in reaction mixtures. Arbitrary density of monomeric Aβ42 bands was calculated in the right column, 5 μL/lane. (c) Aβ concentrations of supernatant and precipitate in the reaction mixtures. AP (100 μg/mL), PC (65 μg/mL), and MN (35 μg/mL) were incubated with Aβ42 (20 μM) for 24 hours and centrifuged to fractionate supernatants and pellets as described in Section 2 (ND: not detected; differences compared to vehicle groups, ***P < 0.001 and *P < 0.05 (Student’s t-test)). Values are the means ± s.e.m., n = 3.
3.2. Dissociative Activity of Procyanidins against Aβ Aggregates. To investigate whether the PC can dissolve aggregated Aβ42, we also performed the Th-T assay using a posttreatment protocol. After the incubation of Aβ42 (20 μM) for 48 hours, various concentrations of AP, PC, and MN were added and then were additionally incubated for 30 minutes (Figure 3(a)). PC effectively dissociated Aβ42 aggregates in a dose-dependent manner (65–650 μg/mL), while AP resulted in limited dissociation (Figure 3(a)). On the other hand, Aβ disassociation was not observed after 360 μg/mL MN treatment (Figures 3(a) and 3(b)). In addition, AP and PC also significantly dissociated Aβ aggregates at 30 minutes, and the dissociation continued until 5 hours after addition of the compounds (Figure 3(b)). In contrast, AP and PC (50 μg/mL and 32.5 μg/mL, resp.) failed to dissociate Aβ aggregates (data not shown). This result indicated that AP and PC dissociated Aβ aggregates in a high-concentration manner.

3.3. Neuroprotective Effects of Procyanidins against Aβ42-Induced Toxicity. Aβ42 plays a pivotal role in the pathogenesis AD because of its potent aggregative ability and neurotoxicity [26]. PC-12 cells were established to measure cellular viability associated with Aβ42 treatment. These cells were used to investigate the neuroprotective effect of PC against Aβ42-induced neurotoxicity using an MTT assay [27]. The Aβ42 (1 μM) induced cytotoxicity (23.7 ± 2.4% viability) in the cells after coincubation for 36 hours (Figure 4(a)). When PC-12 cells were preincubated with AP or PC for 1 hour and then treated with Aβ42 for 36 hours, AP and PC significantly inhibited cytotoxicity in a dose-dependent manner. In particular, 32.5 μg/mL PC restored cell viability to 101.2 ± 5.6% (Figure 4(a)). On the other hand, 100 μg/mL AP restored cell viability to 88.0 ± 13.7% (Figure 4(a)). When the cells were treated with AP or PC alone (without Aβ42) for 36 hours, a beneficial effect on cell viability was observed at concentrations of 32.5 μg/mL PC and 100 μg/mL AP (Figure 4(b)). These results indicated that apple PC prevented Aβ42-induced cytotoxicity on PC-12 cells.
4. Discussion

Polyphenols extracted from functional plants have been reported to show antiaggregative and anti-neurotoxic properties in vitro [28], and it is not clear whether AP has these functions. Polyphenols, however, usually have poor bioavailability, and this is even more marked for macromolecular substances containing PC [29, 30]. On the other hand, the long-term administration of several polyphenols effectively prevents AD-like pathologies and memory impairment in a mouse model of AD [20, 31, 32]. We have also shown that orally silymarin treatment, extracted from milk thistle (Silybum marianum), attenuates AD-like phenotypes in a mouse model of AD [22]. Furthermore, a specific Porter method and high-performance liquid chromatography/tandem mass spectrometry identified apple PC oligomers at a concentration of 11.4 μg/mL in rat plasma 2 hours after single intake of high dose 1,000 mg AP/kg body weight [24]. The present study showed that PC inhibited Aβ aggregation and neurotoxicity at an IC50 of 4.8 μg/mL and 9.4 μg/mL, respectively (Figures 1 and 4), suggesting that apple PC is able to prevent amyloidogenesis at least in vessels. Therefore, it might be able to ameliorate AD-like pathologies and memory impairment in a mouse model of AD receiving long-term administration of PC. Further studies are needed to clarify this matter.

In Figure 1, Th-T analyses on Aβ aggregation indicated that the IC90 of AP was 87.8 μg/mL, while the IC90 of PC was 24.7 μg/mL, which corresponded to 38.0 μg/mL AP (Figure 1(a)). Consequently, the inhibitory activity of PC against Aβ aggregation was approximately 2-fold higher than that of AP. Furthermore, the dissociative effect of AP on Aβ was saturated at concentration of 100 μg/mL, while that of PC continued to increase until 240 μg/mL (Figure 3(a)). These results corresponded to cell viability of MTT assays (Figure 4), suggesting that PCs have a more potent ability to promote Aβ disaggregation and neuroprotection than whole APs. In contrast, MN showed limited depression of Aβ aggregation. Whole AP and MN may contain factors that counteract the antiaggregative and neuroprotective activity of PC.

Hirohata et al. reported that myricetin, an antioxidant polyphenol, exerted an anti-amyloidogenic effect by reversible binding to the Aβ fibril structure in vitro [33]. Furthermore, Kirschner et al. reported that curcumin suppresses Aβ aggregation via hydrogen binding to Aβ42 at Glu11-Gly25 [34]. Kumar et al. also reported a structural analysis demonstrating that curcumin directly binds to Gln15, Glu22, and Asp23 of Aβ42 [35]. The Gln15 to Ile32 region is predicted
to be an intramolecular β-sheet in Aβ assemblies; thus curcumin can disturb Aβ assembly due to binding to Aβ in vitro as well as in vivo [20, 26]. Apple PC binding to Aβ at position Glu15-Ile32 might directly inhibit the conformation of Aβ42. Pasinetti et al. reported that grape seed polyphenolic extract including PC interferes with paired helical filament formation by direct physical intercalation with tau molecules [36]. Therefore, apple PC might have a similar suppressive effect on intermolecular aggregation of tau as well as Aβ42. Interestingly, we also observed a brown-colored pellet that formed following treatment of Aβ42 aggregates with PC and AP (not data shown), suggesting that PC could bind to Aβ42 aggregates. On the other hand, treatments with neither vitamin-C (2 mM) nor the potent antioxidative agent, EUK-134 (40 μM), did not suppress the Aβ aggregation (data not shown), thus suggesting that the antiaggregative activity of PC might likely be independent of its antioxidant properties. Although Yatin et al. reported that vitamin-E (VE) does not suppress Aβ42 aggregation [37], Yang et al. revealed that α-tocopherol quinone derived from VE inhibits Aβ aggregation in a dose-dependent manner [38]. These findings suggest that the chemical structure of these compounds impacts the inhibition of Aβ aggregation rather than their antioxidative capacity.

In Figure 4(a), PC showed potent neuroprotective effects in a dose-dependent fashion, demonstrating that PC can play a role in both Aβ-disaggregation and cellular survival. In addition, the neuroprotective effect of PC was stronger than that of AP as well as MN (Figure 4(a)), which was consistent with the antiaggregative activity of PC (Figures 1 and 3), suggesting that PC suppress Aβ aggregation leading to neuroprotection against Aβ42. Interestingly, we also found an additional effect of PC on cell viability analysis. When PC-12 cells were treated with PC (32.5 μg/mL) without Aβ, the percentages of cell viability were enhanced to over 100% (Figure 4(b)). Furthermore, AP also significantly induced cellular viability at a concentration of 100 μg/mL (Figure 4(b)). Miura et al. previously reported that treatment with low-dose PC enhanced cell proliferation, while high-dose PC induced apoptosis in melanoma cells [16]. Recently, Choi et al. reported that PC treatment inhibits endogenous histone acetyltransferase, subsequently suppresses cell proliferation, and increases cell death in prostate cancer cells [39]. These results suggest that PC can regulate cell proliferation in a dose-dependent manner. Therefore, apple PC might not only suppress Aβ aggregation but also modify neuronal cell proliferation, thus contributing to the neuroprotective effect against Aβ-induced cytotoxicity.

In conclusion, apple PC acted as a potent suppresser of abnormal Aβ aggregation and a showed protective effect on neuronal survival in vitro. Since apple PC is a safe and an inexpensive food factor, it might be promising for long-term treatment. Our study suggests a novel activity of apple PC, further supporting the consideration of their use for either the prevention or treatment of Aβ aggregation associated with neurodegenerative disorders.

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References


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