Roasted Coffee Reduces $\beta$-Amyloid Production by Increasing Proteosomal $\beta$-Secretase Degradation in Human Neuroblastoma SH-SY5Y Cells

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Scope: Epidemiological studies have shown that coffee consumption may be associated with a lower risk of developing several neurological disorders, including Alzheimer’s disease (AD). Caffeine is a prominent candidate component underlying the preventive effects of coffee; however, the contribution of other constituents is unclear. To clarify this issue, the effect of roasting coffee beans on $\beta$-secretase (BACE1) expression in human neuroblastoma SH-SY5Y cells is investigated.

Methods and results: Coffee (2%) reduces $A\beta$ accumulation in culture medium to 80% of control levels after 24 h. Accordingly, BACE1 expression is decreased to 70% of control levels at 12 h. Experiments using cycloheximide and MG132, a proteasome inhibitor, reveal that coffee enhanced BACE1 degradation through activation of proteosomal activity. Furthermore, coffee activates cAMP-dependent protein kinase, and consequently, phosphorylation of a serine residue of proteasome 26S subunit, non-ATPase 11 (PSMD11). Pyrocatechol, a strong antioxidant known as catechol or 1,2-dihydroxybenzene, produced from chlorogenic acid during roasting, also reduces BACE1 expression by activation of proteosomal activity. Furthermore, pyrocatechol reduces $A\beta$ production in SH-SY5Y cells.

Conclusion: The data suggest that the roasting process may be crucial for the protective effects of coffee consumption in AD.

1. Introduction

Coffee is a world-wide consumed beverage. Epidemiological studies show that coffee consumption may be associated with a lower risk for several neurological disorders, such as Alzheimer’s disease (AD) and Parkinson’s disease.[1–3] Several cross-sectional and longitudinal population-based studies have suggested a protective effect of coffee use against late-life cognitive impairment and decline.[1] However, elucidation of the molecular mechanisms for the protective effects of coffee against these neurological disorders have not been fully explored.

Genetic and experimental evidence implicate the accumulation of $\beta$-amyloid ($A\beta$) in the brain and the toxicity of $A\beta$ oligomers in the etiology of AD.[4] $A\beta$ peptides are generated from the cleavage of amyloid precursor protein (APP) by the enzymes $\beta$- and $\gamma$-secretases.[5] Inhibition of these secretases represents an obvious logical strategy to inhibit the generation of $A\beta$. However, $\gamma$-secretase not only cleaves APP but many other type I transmembrane proteins within their transmembrane domains including Notch receptors.[6] Therefore, long-term treatment of AD patients with $\gamma$-secretase inhibitors appears unfeasible due to side effects.[8] On the other hand, the $\beta$-site APP-cleaving enzyme 1 (BACE1) is the major protease with $\beta$-secretase activity in the brain and is thought to initiate the amyloidogenic pathway to cleave APP to form the N-terminus of $A\beta$ peptides.[5,7] Therefore, the development of selective BACE1 inhibitors has been pursued in academia and industry.[8,9] However, recent clinical trials of BACE1 inhibitors have failed to rescue the cognitive decline in patients with mild-to-moderate AD, indicating that treatment at the symptomatic stage might be too late.[10] In these circumstances, elucidation of the molecular basis of effects of the coffee on AD is an urgent issue, because the safety and efficacy of coffee intake of treating AD progression is highly expected.[11]

Arendash et al. reported that caffeine protected AD mice against cognitive impairment and reduced $A\beta$ production by the reduction of $\beta$- and $\gamma$-secretase activities.[12,13] Although caffeine is the most prominent constituent of coffee, and a likely candidate for its preventive effect against AD.[12–14] recent studies revealed that the therapeutic benefits of coffee are not...
due to caffeine alone.[14–16] Shukitt-Hale et al. showed that improvements in cognition and psychomotor behavior in aged rats were due to coffee, and not caffeine itself.[17] Furthermore, crude caffeine, a by-product of the coffee decaffeination process, exhibited greater therapeutic effect on memory impairment in AD mice than caffeine alone.[18] This study also reported that administration of crude caffeine reduced the Aβ levels and the number of amyloid plaques in the hippocampus in AD mice.[18] To elucidate the molecular basis of the preventive effect of coffee against AD, we focused on the effects of coffee on BACE1 expression in human neuroblastoma SH-SY5Y cells by using coffee beans with different degrees of roasting.

2. Experimental Section

2.1. Materials

SH-SY5Y cells were purchased from DS Pharma Biomedical Co. Ltd (Osaka, Japan). Reagents for PCR were purchased from Applied Biosystems Inc. (Warrington, UK). Antibodies for BACE1, phospho-serine, and PSMD11 were purchased from Abcam (Cambridge, UK), QIAGEN (Hilden, Germany) and ATGenCo., Ltd (Gyeongi-do, South Korea), respectively. The antibody for β-actin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal antibodies for Aβ1–40 end-specific (4D1), and Aβ1–40 end-specific antibody (4D8) were prepared as previously described.[19] Caffeine, caffic acid, chlorogenic acid, chloroquine, and trigonelline were purchased from Sigma (St. Louis, MO). MG132 and H89 were purchased from Nacalai Tesque (Tokyo, Japan).

2.2. Cell Culture and Treatment with Coffee or Reagents

SH-SY5Y cells were grown in Ham’s F12/DMEM medium (1:2). CellCultureandTreatmentwithCoffeeorReagents

2.3. Preparation of Coffee Extract

Roasted coffee powder (Columbia Arabica) was obtained from Starbucks Coffee Japan (Tokyo, Japan). Brewed coffee was prepared by a common method, in which 8 g of powder was extracted with 140 mL hot water (95 °C) and filtered through a paper filter (Mellita, Minden, Germany). The brewed coffee was then steril filtered, divided into small aliquots, and stored at −80 °C until used. Undiluted brewed coffee, with a dry weight of 8.4 mg mL−1, was assigned a concentration of 100% (v/v). For roasting experiments, Columbian Arabica coffee green beans were purchased from Nakaya Coffee (Tokyo, Japan), and the green beans were roasted at 200—220 °C for up to 20 min. Green and roasted beans were ground, and then coffee extracts were prepared and stored as described above. Optical densities at 500 nm of 10% brewed coffee made from the roasted beans were 0.05 (green beans), 0.153 (medium roasted beans), and 0.269 (dark roasted beans).

2.4. Measurement of Aβ40 Level in Cell Supernatants

Cells were treated with coffee (0–2%) for 24 h; the culture supernatant was collected. The amount of Aβ40 in the supernatants was determined by ELISA, according to the method described previously.[19] Briefly, 96-well plates were coated with the monoclonal Aβ1–40 end-specific antibody (4D1) (0.3 μg per well), washed with PBS containing 0.05% (v/v) Tween 20 (washing buffer, WB), blocked with bovine serum albumin (3% (w/v) in PBS), washed with WB. The supernatant (100 μL) was then incubated together with a standard of synthetic Aβ1–40 peptide (gifted from the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University) for one night at 4 °C. After washing, wells were treated with the biotinylated antibody for Aβ1–40 (2D1) (12.5 ng), washed, and incubated with 100 μL of a streptavidin–horseradish peroxidase complex (1:5000) (GE Healthcare Bio-Science Japan, Tokyo, Japan). The plate was further washed, and 100 μL of TMB peroxidase substrate solution (Agilent Technologies, Santa Clara) was added to wells and incubated for 20 min at room temperature. The reaction was stopped by the addition of 100 μL of 0.2 M sulfate, and the absorbance at 450 nm was measured.

2.5. Analysis of Gene Expression

Total RNA was isolated from the cultured cells by the guanidinium thiocyanate-phenol-chloroform extraction method using Isogen (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 1 μg of total RNA using 100 units of reverse transcriptase and random primers according to the manufacturer's protocol (TOYOBO Life Science, Osaka, Japan). Quantitative real-time PCR (qPCR) was performed using the SYBR green PCR core reagent kit (Roche, Basel, Switzerland) according to the manufacturer’s protocol. The primers used for the amplification of BACE1 cDNA were designed based on the published sequences as follows (forward and reverse, 5’ to 3’): CACCACCAACCTTGTCTTCGC and TTAGGTAGAGTGA-GATGACTGGGAAA.

2.6. Immunoprecipitation and Immunoblotting

Cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM Na2HPO4, 1 mM EDTA, 0.5% Nonidet P-40, phosphatase inhibitor cocktail (Nacalai Tesque), and protease inhibitor cocktail (Nacalai Tesque). Crude cell extracts were centrifuged at 20,000 × g for 10 min, and proteins (10 μg) were...
resolved by SDS-PAGE and transferred onto PVDF membrane (Millipore, Billerica, MA). The membrane was incubated for 1 h at room temperature in blocking buffer consisting of TBS (20 mM Tris-HCl pH 7.4, 137 mM NaCl) containing 5% skim milk. The membrane was then incubated overnight at 4 °C with the designated primary antibody. The membrane was further incubated with horseradish peroxidase-linked secondary antibodies (Agilent Technologies, Santa Clara). Immunoreactive proteins were visualized with the ECL detection system (GE Healthcare Bio-Science Japan, Tokyo, Japan).

2.7. Immunoprecipitation Analysis

Cell lysates were incubated with anti-PSMD11 antibody and then protein G-Sepharose (Zymed Laboratory, South San Francisco, CA, USA) at 4 °C for 4 h. Immune complexes were precipitated and washed five times with lysis buffer, then eluted with Laemmli buffer. Eluted proteins were resolved using SDS–PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were probed using the anti-phosphoserine antibody (Abcam) and visualized with the ECL detectionsystem.

2.8. Assay for Proteasome Activity in SH-SY5Y Cells

Proteasome activity within the cells was measured using Proteasome 20S Activity Assay Kit (Sigma–Aldrich, St. Louis, MO) according to the manufacturer’s protocol, with minor modifications. In brief, 3 × 10⁵ cells were plated in a 96-well plate and incubated overnight at 37 °C in a humidified atmosphere of 5% CO₂. Cells were treated with coffee or other compounds in 100 μL protease assay loading buffer containing 1 μg LLVY-R110 substrate, then the fluorescence intensity generated by proteasome cleavage of the substrate was measured at λex = 490 nm and λem = 525 nm with a plate reader (Infinite 200, TECAN Japan, Tokyo, Japan) after 4 h.

2.9. Extraction and Separation of Coffee Extracts

To extract coffee constituents with organic solvents, brewed coffee (1 mL) was extracted sequentially with an equal volume of hexane, ethyl acetate, and n-butanol, sequentially. Organic and aqueous phases were then concentrated by evaporation, and the concentrates were dissolved in 20 μL DMSO. To separate ethyl acetate extracts, dried ethyl acetate extracts (equivalent to 15 mL coffee) were dissolved in 15 mL of methanol/ethyl acetate (2:1), applied to Sep-Pak C₁₈ column (Waters, Milford, MA), and eluted by 15 mL step-wise rinses of water (R1), 20% methanol (R2), and 100% methanol (R3).

2.10. High-Performance Liquid Chromatography of Coffee Extracts

High-performance liquid chromatography (HPLC) analyses were performed using an ODS column (Inertsil ODS-3 column, 4.6 × 250 mm, GL Sciences Inc., Tokyo, Japan) at 40 °C. Coffee extracts or standards were applied to the column and eluted with the solvent A (5% acetate) to B (100% methanol) gradient system at a flow rate of 1 mL min⁻¹, detected with fluorescence at λex = 280 nm and λem = 320 nm.

2.11. Statistics

Student’s t-tests were used for statistical analyses and p < 0.05 was considered significant.

3. Results

3.1. Coffee Reduces Aβ Production in SH-SY5Y Cells

To explore the effect of coffee on Aβ production in neural cells, human neuroblastoma SH-SY5Y cells were treated with coffee, and secreted Aβ protein in the media was measured. Since in our conditions, treatments with less than 2.5% (v/v) coffee did not exhibit any cytotoxicity as determined using trypan blue staining and WST assay (data not shown), we measured the effect of coffee preparations of less than 2% (v/v). After 24 h incubation with coffee (0–2%), the amount of Aβ₄₀ in the media was determined by sandwich ELISA as described.[19] The Aβ₄₀ amounts decreased in a dose-dependent manner, reaching ~20% of initial level following treatment with 2% coffee (Figure 1). The amount of Aβ₄₂ was too low to detect by our methods.

3.2. Coffee Reduces BACE1 Expression in SH-SY5Y Cells

In the amyloidogenic pathway, BACE1 cleaves APP at the β-secretase site to initiate the production of Aβ peptides, and this cleavage constitutes the rate-limiting step.[14] Therefore, we measured the effect of coffee on the expression of BACE1 protein...
Coffee reduces BACE1 expression in SH-SY5Y cells. Cells were treated with 2% coffee for the indicated times and harvested. A) Cell lysates (10 µg) were subjected to immunoblotting for BACE1 and β-actin, and B) the band intensities of BACE1 were quantified with an image analyzer. C) RNA was isolated from the harvested cells and BACE1 mRNA levels were measured by real-time PCR. D) Cells were treated with different concentrations of coffee ranging from 0 to 2% for 15 h and the BACE1 expression was measured as in (A) and (B). *p < 0.05, **p < 0.01 (n = 3).

3.3. Coffee Enhanced BACE1 Degradation in SH-SY5Y Cells

To determine whether coffee affects the BACE1 degradation, cells were treated with cycloheximide (CHX) to inhibit de novo protein synthesis, and BACE1 expression was measured in cells with or without coffee treatment. Although initial rates of BACE1 degradation were similar, BACE1 degradation was enhanced in cells treated with coffee after 6 h incubation (Figure 3A).

Since it has been reported that BACE1 is degraded by the lysosomal pathway,[20] we applied chloroquine, an inhibitor of lysosomal proteases, in addition to cycloheximide for the measurement of BACE1 degradation. Although chloroquine slightly delayed the degradation rate, the addition of coffee significantly enhanced the rate of BACE1 degradation after 6 h incubation (Figure 3B). It has also been reported that the degradation of BACE1 is regulated by the ubiquitin-proteasome system (UPS), the major intracellular protein quality control system in eukaryotic cells.[21] To elucidate the effect of coffee on the UPS-dependent BACE1 degradation, we analyzed the effect of MG132, a proteasome inhibitor, on the enhancement of BACE1 degradation by coffee. As shown in Figure 3C, MG132 attenuated the coffee-mediated reduction in BACE1 protein expression.

3.4. Coffee Induced Proteasomal Activity via Protein Kinase A Activation in SH-SY5Y Cells

To elucidate the molecular basis for the coffee-mediated activation of UPS, we measured proteasomal activity in coffee-treated SH-SY5Y cells using a synthetic substrate LLVY-R110. As shown in Figure 4A and B, proteasomal activity was induced by coffee treatment in a dose-dependent manner. Since it was reported that proteasome function is regulated by cyclic AMP-dependent protein kinase A (PKA) through the phosphorylation of PSMD11 (proteasome 26S subunit, non-ATPase 11)—one of 19S proteasome subunits[22]—we measured the effect of PKA inhibitor H89 on the coffee-mediated activation of proteasome activity. As shown in Figure 4B, H89 blocked the coffee-mediated activation of proteasome activity. To further investigate the effect of coffee on the phosphorylation of PSMD11 induced by PKA, SH-SY5Y cells were treated with coffee in the presence and absence of H89. PSMD11 was then immunoprecipitated with anti-PSMD11 antibody and then immunoblotted with anti-phosphoserine antibody. Strikingly, coffee induced phosphorylation of PSMD11, and this phosphorylation was blocked with H89, suggesting that coffee induced the PKA-mediated PSMD11, leading to the activation of proteasomes (Figure 4C).

3.5. Analyses of Coffee Constituents Responsible for the Reduction in BACE1 Expression

There are several major constituents in coffee extracts, which exhibit specific physiological activities. These include caffeine, caffeic acid, chlorogenic acid, and trigonelline (Figure 5A). To explore which coffee constituent is responsible for the reduction in BACE1 expression in coffee-treated SH-SY5Y cells, we measured the effects of major coffee components at 100 µM (the concentration equivalent to 2–5% coffee for all components). No compound exhibited reduction in BACE1 expression (Figure 5A).

Although caffeine is recognized as the most prominent constituent for the preventive effect of coffee against AD,[14] no reduction was observed with caffeine treatment. Therefore, we examined the effect of decaffeinated coffee on BACE1 protein.
expression. Decaffeinated coffee contains only a trace amount of caffeine (<1/100 of the original coffee; unpublished data). Nonetheless, 2% decaffeinated coffee treatment significantly reduced BACE1 expression, although the magnitude of the reduction was slightly weaker than that observed following treatment with normal coffee (Figure 5B). Furthermore, decaffeinated coffee also enhanced proteasomal activity, and the enhancement was attenuated by H89 (Figure 5C).

To examine the possibility that active components are formed during the roasting process, we assayed the activity of extracts from beans that had undergone varying degrees of roasting before brewing (Figure 6A). As shown in Figure 6B, coffee prepared from the roasted beans (medium and dark) but not from the green beans reduced BACE1 expression in treated cells. To further characterize active coffee constituents, coffee brewed from dark roasted beans was sequentially extracted with equal volume of n-hexane, ethyl acetate, and n-butanol, and the effect of each extract on BACE1 expression was measured. Activity to reduce BACE1 expression was only detected in the ethyl acetate extract (Figure 6C).

3.6. Pyrocatechol Reduced BACE1 Expression via the Activation of Proteasomal Activity

In order to characterize the components responsible for the BACE1 reduction, we performed HPLC analysis using a reverse phase column. The analysis of the coffee extracts revealed that two major substances, pyrocatechol and 4-ethyl catechol, were produced during roasting process (Figure 7A). These two substances were separated by Sep-Pak C18 column chromatography and the major BACE1 expression reducing activity was recovered in the fraction containing pyrocatechol (R2) (Figure 7B). Since the dark coffee extract contained 2.5 µM pyrocatechol at 2% (v/v), we measured the effect of pyrocatechol on BACE1 expression in SH-SY5Y cells up to 10 µM, a concentration at which no cytotoxicity was observed. After 12 h incubation, 2.5 µM pyrocatechol reduced BACE1 expression to half of the control level (Figure 8A). Pyrocatechol also induced proteasomal activity and the effect was inhibited by H89 (Figure 8B). Pyrocatechol strongly activated serine phosphorylation of PSMD11 (Figure 8C). In addition, pyrocatechol reduced Aβ secretion into the medium, as was observed...
Figure 4. Coffee-induced proteasomal activity via protein kinase A activation in SH-SY5Y cells. A) Cells were plated in a 96-well plate and treated with or without 2% coffee in 100 µL assay buffer containing LLVY-R110 as a substrate. At indicated times, the fluorescence intensity generated by 26S proteasome cleavage of the substrate was monitored at λ<sub>ex</sub> = 490 nm and λ<sub>em</sub> = 525 nm. B) Cells were incubated with 0–2% coffee or 2% coffee and 10 µM H89 for 4 h. Proteasomal activities were measured as in (A). C) Cells were treated with 2% coffee ± 10 µM H89 for 4 h and cell lysates were prepared. The lysates (500 µg) were immunoprecipitated with 1 µg anti-PSMD11 antibody. Immune complexes were recovered with Protein G-Sepharose beads and subjected to immunoblot analyses using anti-phosphoserine antibody. Band intensities of p-Ser were quantified. *p < 0.05, **p < 0.01 (n = 3).

Figure 5. Analyses of coffee constituents for the reduction in BACE1 expression. A) Cells were incubated with 2% coffee or 100 µM major constituents, caffeine, caffeic acid, chlorogenic acid, or trigonelline, for 15 h. BACE1 expression were monitored by immunoblotting. Ctrl, control (0.1% DMSO). B) Cells were treated with decaffeinated coffee (0.5–2%) for 15 h and BACE1 expression was measured. C) Cells were incubated with 0–2% decaffeinated coffee or 2% coffee and 10 µM H89 for 4 h. Proteasomal activities were measured as in Figure 4. *p < 0.05, **p < 0.01 (n = 3).
Recent studies have shown that therapeutic benefits of coffee are not due to caffeine alone.\textsuperscript{15,17,18} This suggests that there may be potentiating effects from other unknown coffee components on caffeine, leading to its benefits in preventing AD. Our data...
Figure 7. Characterization of coffee extracts with HPLC. A) HPLC of coffee extracts brewed from green beans and dark roast beans using an ODS column detected with fluorescence at $\lambda_{ex} = 280$ nm and $\lambda_{em} = 320$ nm. B) HPLC of fractions (R1, R2, and R3) separated with Sep-Pak C18 from ethyl acetate extracts of dark roasted coffee as described in the Experimental Section (upper). Cells were treated with each fraction for 15 h and the BACE1 expression was monitored (lower). Arrows indicate the position of standards as follows: arrow 1, pyrocatechol; arrow 2, caffeine; arrow 3, 4-ethyl catechol.

Figure 8. Pyrocatechol reduced BACE1 expression via activation of proteasomal activity in SH-SY5Y cells. A) Cells were treated with 0–10 $\mu$m pyrocatechol for 15 h and then the cell lysates were subjected to immunoblot for BACE1 (upper) and quantified (lower). B) Cells were treated with 2% coffee, 10 $\mu$m pyrocatechol and 100 $\mu$m caffeine for 15 h in the presence or the absence of H89. BACE1 expression was detected by immunoblot (upper) and measured (lower). C) Cells treated with 10 $\mu$m pyrocatechol for 15 h in the presence or the absence of H89. Cell lysates were subjected to the assay for p-Ser in PSMD11 as described in Figure 4C. D) Cells were treated as in (B) and the Aβ1-40 accumulation in the medium was measured as described in Figure 1. *$p < 0.05$, **$p < 0.01$ ($n = 3$).
support this, although caffeine had no effect on BACE1 expression in our experiments. Our results suggest that pyrocatechol, known as a strong antioxidant, is a promising candidate as the active compound in coffee to protect the brain in AD. It is not clear at the moment whether the antioxidant activity of pyrocatechol is a key function for the preventive action of coffee, however, it is tempting to use the active component as a supplement for prophylactic intervention far before surfacing of AD symptoms in future. For this purpose, elucidation of the effectiveness and safety of pyrocatechol using animal models is required.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

Alzheimer’s disease, Aβ, β-secretase, coffee, SH-SYSY