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journal or publication title	博士論文本文Full
year	2015-09-28
学位授与番号	13301甲第4284号
学位名	博士（医学）
学位授与年月日	2015-09-28
URL	http://hdl.handle.net/2297/44640

doi: <https://doi.org/10.1111/jnc.13180>

Original Article

Phenolic compounds prevent the oligomerization of α -synuclein and reduce synaptic toxicity

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Running title: Phenolic compounds prevent α S oligomerization

Keywords: α -synuclein, phenolic compounds, oligomerization, α -synucleinopathies, myricetin, rosmarinic acid

Abbreviations used: atomic force microscope, AFM; α -synuclein, α S; α -synuclein fibrils; α S; circular dichroism spectroscopy, CD; curcumin, Cur; dementia with Lewy bodies, DLB; electron microscope, EM; epigallo catechin 3-gallate, EGCG; ferulic acid, FA; field excitatory postsynaptic potentials, fEPSPs; glutathione S-transferase, GST; Lewy body; LB; long-term potentiation, LTP; matrix-assisted laser desorption ionization time-of-flight mass, MALDI-TOF MS; 1-methyl-4phenyl-1,2,3,4-tetrahydropyridine, MPTP; myricetin, Myr; nordihydroguaiaretic acid, NDGA; nuclear magnetic resonance, NMR; Parkinson's disease, PD; photo induced cross-linking of unmodified proteins, PICUP; rosmarinic acid, RA; size-exclusion chromatography, SEC; sodium dodecyl sulfate-polyacrylamidegel electrophoresis, SDS-PAGE; thioflavin S, ThS; thioflavin T, ThT

Abstract

Lewy bodies, mainly composed of α -synuclein (α S), are pathological hallmarks of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Epidemiological studies showed that green tea consumption or habitual intake of phenolic compounds reduced PD risk. We previously reported that phenolic compounds inhibited α S fibrillation and destabilized preformed α S fibrils. Cumulative evidences suggest that low-order α S oligomers are neurotoxic and critical species in the pathogenesis of α -synucleinopathies. To develop disease modifying therapies for α -synucleinopathies, we examined effects of phenolic compounds (myricetin (Myr), curcumin, rosmarinic acid (RA), nordihydroguaiaretic acid, and ferulic acid) on α S oligomerization. Using the methods of photo induced cross-linking of unmodified proteins, circular dichroism spectroscopy, the electron microscope, and the atomic force microscope, we showed that Myr and RA inhibited α S oligomerization and secondary structure conversion. The nuclear magnetic resonance analysis revealed that Myr directly bound to the N-terminal region of α S, on the other hand, direct binding of RA to monomeric α S was not detected. Electrophysiological assays for long-term potentiation in mouse hippocampal slices revealed that Myr and RA ameliorated α S synaptic toxicity by inhibition of α S oligomerization. These results suggest that Myr and RA prevent α S aggregation process and reduce the neurotoxicity of α S oligomers.

Introduction

Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) belong to the category of α -synucleinopathies. PD is characterized by the loss of nigrostriatal dopaminergic neurons associated with motor impairment, and the main symptoms of PD are muscle rigidity, resting tremor, bradykinesia, and postural instability (Jankovic 2008). In PD, neurodegenerative lesions with Lewy bodies (LBs) are distributed mainly in the dopaminergic neurons of the substantia nigra, and also in other brain stem and limbic regions (Braak *et al.* 2004). DLB is a disorder of progressive dementia characterized by fluctuation in mental decline, visual hallucination, parkinsonism, and widespread distribution of LBs in the brain (McKeith *et al.* 2005). LBs are the accumulation of intracellular inclusions of the insoluble protein, which are mainly composed of α -synuclein (α S), and are pathological hallmarks of diseases known as PD and DLB (McKeith *et al.* 2005; Spillantini *et al.* 1998).

α S is a neuronal presynaptic protein, involved in vesicular trafficking, neurotransmitter release, and regulation of neurotransmission (Clayton and George 1999; Fujiwara *et al.* 2002). The monomeric α S is natively unfolded soluble protein without well-defined secondary or tertiary structures (Weinreb *et al.* 1996), but it transforms into cross- β -sheet rich amyloid by self-assembly at physiological conditions via partially folded intermediates and soluble oligomers (Uversky *et al.* 2001). While

the intimate origin for the α S toxicity is yet to be unclear, accumulated evidences suggest that oligomeric forms of α S, rather than the larger intracellular inclusions, might be more bioactive and, possibly, cytotoxic, causing not only neuronal dysfunction but cell death (Martin *et al.* 2012; Winner *et al.* 2011).

Epidemiological studies showed an inverse relationship between green tea consumption and the risk of developing PD (Ascherio *et al.* 2001; Chan *et al.* 1998). The major polyphenols present in green tea are catechins, especially epigallo catechin 3-gallate (EGCG), and green tea contains more myricetin (Myr) compared with black tea (Bosetti *et al.* 2005). Recently, a prospective study showed habitual intake of some polyphenols may reduce PD risk, and the association was more pronounced in men than women (Gao *et al.* 2012).

Evidences of *in vitro* and *in vivo* studies have indicated the protective effects of polyphenols, such as EGCG in green tea, curcuminoids in curry, baicalein extracted from the root of *Scutellaria baicalensis*, a traditional Chinese herb, or extracts from grape and blueberry, against neuronal damage in PD (Chao *et al.* 2012; Masuda *et al.* 2006). Levites *et al.* reported the neuroprotective activity of EGCG on 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP)-induced parkinsonism in animal models (Levites *et al.* 2001). It is suggested that the neuroprotective effects of EGCG are mediated by iron-chelating activities and free-radical-scavenging activities possessed by the cathecol group (Weinreb *et al.* 2009). Polyphenols also have

protective effects against α S toxicity (Liu *et al.* 2011; Jiang *et al.* 2013). In PD cell model experiments, Curcumin (Cur) reduced α S induced cytotoxicity by reduction of intracellular reactive oxygen species, mitochondrial depolarization, cytochrome c release, and caspase-9 and caspase-3 activation (Liu *et al.* 2011), or downregulation of mTOR/p70S6K signaling and the recovery of macroautophagy (Jiang *et al.* 2013).

We showed that phenolic compounds such as the wine-related polyphenol Myr, a major component of curry spice turmeric Cur, rosmarinic acid (RA), nordihydroguaiaretic acid (NDGA), and ferulic acid (FA) inhibited the formation of α S fibrils (f α S), as well as destabilized preformed fibrils (Ono and Yamada 2006). Similarly, it was reported that baicalein (Zhu *et al.* 2004) and EGCG (Bieschke *et al.* 2010; Ehrnhoefer *et al.* 2008) also inhibited f α S formation and destabilized preformed f α S.

Given this background, we examined the ability of five phenolic compounds, Myr, FA, NDGA, Cur, and RA (Fig.1), to interact with α S and to inhibit the oligomerization of α S using well-established biophysical techniques. We assessed the oligomerization of α S with the methods of photo induced cross-linking of unmodified proteins (PICUP), sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE), circular dichroism spectroscopy (CD), electron microscope (EM), atomic force microscope (AFM), and nuclear magnetic resonance (NMR) (Bitan and Teplow 2004; Ono *et al.* 2008; Ono and Yamada 2011). We also evaluated the seeding effect of oligomeric α S

using thioflavin S (ThS) assay (LeVine 1993; Naiki and Nakakuki 1996; Ono *et al.* 2013). Finally, we examined whether the phenolic compounds reduced α S oligomers-induced synaptic dysfunction with electrophysiological assays for long-term potentiation (LTP).

Materials and methods

Chemicals and reagents

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest purity available. Water was produced using a Milli-Q system (Nihon Millipore K.K., Tokyo, Japan).

Proteins and phenolic compounds

α S peptides were obtained from r-peptide (Osaka, Japan), and were >95% purity. Purified peptides were stored as lyophilizates at -20°C. To prepare peptides for study, α S peptide lyophilizates were dissolved at a nominal concentration of 50 μ M in 20 mM Tris HCl buffer, pH 7.4. The peptide solution was centrifuged for 30 min at 16,000 $\times g$ at 4°C. A stock solution of glutathione S-transferase (GST; ~26 kDa) (Sigma-Aldrich) was prepared by dissolving the lyophilizate to a concentration of 250 μ M in 60 mM NaOH. Prior to use, aliquots were diluted 10-fold into 20 mM Tris-HCl, pH 7.4. We examined 5-phenolic compounds such as Myr, FA, NDGA, Cur, and RA. They were

dissolved in ethanol to a final concentration of 2.5 mM and then diluted with 20 mM Tris HCl buffer, pH7.4, to produce concentrations 5, 10, 25, 50, 100, and 500 μ M for CD, PICUP, and AFM as described previously (Bitan and Teplow 2004; Ono *et al.* 2012a).

Circular dichroism spectroscopy

CD spectra of α S compound mixtures were acquired immediately after sample preparation or following 1-5 days of incubation. CD measurements were made by removing a 200- μ L aliquot from the reaction mixture, adding the aliquot to a 1-mm path length CD cuvette (Hellma, Forest Hills, NY), and acquiring spectra in a J-805 spectropolarimeter (JASCO, Tokyo, Japan). The CD cuvettes were maintained on ice prior to introduction into the spectrometer. Following temperature equilibration, spectra were recorded at 22°C from ~190-260 nm at 0.2 nm resolution with a scan rate of 100 nm/min. Ten scans were acquired and averaged for each sample. Raw data were manipulated by smoothing and subtraction of buffer spectra according to the manufacturer's instructions.

Chemical cross-linking and determination of oligomer frequency distributions

Immediately after their preparation, samples were cross-linked using PICUP, as described (Ono *et al.* 2012b). Briefly, to 18 μ M of 50 μ M protein solution were added

1 μ L of 4 mM tris(2,2'-bipyridyl)dichlororuthenium(II) (Ru(bpy)) and 1 μ L of 80 mM ammonium persulfate. The final protein of α S : Ru(bpy) : ammonium persulfate molar ratios were 1 : 4 : 80. The mixture was irradiated for 1 s with visible light, and then the reaction was quenched with 2 μ L of 1 M DTT (Invitrogen) in ultrapure water. Determination of the frequency distribution of monomers and oligomers was accomplished using SDS-PAGE and silver staining as described (Ono *et al.* 2012b). Briefly, 8 μ L of each cross linked sample was electrophoresed on a 10-20% gradient tricine gel and visualized by silver staining (Invitrogen). Uncross-linked samples were used as controls in each experiment. Densitometry was performed with a luminescent image analyzer (LAS 4000 mini, Fujifilm, Tokyo, Japan) and image analysis software (Multi gauge, version 3.2, Fujifilm). The intensity of each band in a lane from the SDS gel was normalized to the sum of the intensities of all the bands in that lane according to the formula, $R_i = I_i / \sum I_i \times 100$ (%), where R_i is the normalized intensity of band I_i , and I_i is the intensity of each band i . R_i varies from 0-100. To calculate the oligomer ratio, the sum of oligomers intensities of α S with 2.5, 5, 25, 50, and 250 μ M Myr, FA, NDGA, Cur, or RA, respectively, was divided by the sum of oligomer intensities without each compound. The EC₅₀ was defined as the concentration of phenolic compounds to inhibit α synuclein oligomerization to 50% of the control value. The EC₅₀ was calculated by sigmoidal curve fitting, using GraphPad Prism software (version 4.0a, GraphPad Software, Inc.).

Size-exclusion chromatography

PICUP reagents and phenolic compounds were removed from cross-linked samples by size-exclusion chromatography (SEC) as described previously (Ono *et al.* 2012a; Volles *et al.* 2001). To do so, we used the Superdex 200 10/300 GL column (GE healthcare, Tokyo, Japan). At first, the column was washed twice with 0.5 M NaOH. Two hundred μ L of cross-linked sample was then loaded. The column was eluted with 20 mM Tris HCl buffer at a flow rate of 0.5 mL/min. The first 4 mL of elute was collected. Fractions were lyophilized immediately after collection.

Seeding activity of assemblies of α S

For the seeding assay, uncross-linked α S or cross-linked α S with or without Myr and RA at a concentration of 25 μ M in 20 mM Tris buffer, pH 7.4 were added as seeds to uncross-linked α S at a ratio of 10% (v/v). The mixtures were incubated at 37°C for 0-7 days. ThS fluorescence were measured as mentioned below at 0, 1, 2, 6, 24, 48, 72, 96, 120, 144, and 168 hrs. Before the seeding assay, secondary structure of each α S assembly was checked, using CD studies.

Thioflavin S binding

Thioflavin binding assays were performed because fluorescence intensities do

correlate with α S fibril content (LeVine 1993; Naiki and Nakakuki 1996). The reaction mixture contained 5 μ M ThS (MP Biomedicals, Irvine, CA, USA) and 50 mM glycine-NaOH buffer, pH 8.5. After brief vortexing, fluorescence was determined three times at intervals of 10 s using a Hitachi F-7500 fluorometer (Hitachi, Tokyo, Japan). Excitation and emission wavelength of 440 and 521 nm were used for α S assay, respectively. Fluorescence was determined by averaging three readings and subtracting the ThS blank readings.

Electron microscope

A 10- μ L aliquot of each sample was spotted onto a glow-discharged, carbon-coated formvar grid (Okenshoji, Co, Ltd, Tokyo, Japan) and incubated for 20 minutes. The droplet then was displaced with an equal volume of 2.5% (v/v) glutaraldehyde in water and incubated for an additional 5 minutes. Finally, the peptide was stained with 8 μ L of 1% (vol/vol) filtered (0.2 μ m) uranyl acetate in water (Wako Pure Chemical Industries, Ltd, Osaka, Japan). This solution was wicked off and then the grid was air-dried. The samples were examined using a JEM-1210 transmission EM (JEOL Ltd., Tokyo, Japan).

Atomic force microscope

Peptide solutions were characterized using a Nanoscope IIIa controller (Veeco Digital

Instruments, Santa Barbara, CA, USA) with a multimode scanning probe microscope equipped with a JV (J-type vertical) scanner. All measurements were carried out in the tapping mode under ambient conditions using single-beam silicon cantilever probes. A 10- μ L aliquot of each sample was spotted onto freshly cleaved mica (Ted Pella, Inc., Redding, CA, USA), incubated at room temperature for 5 minutes, rinsed with water, and then blown dry with air. At least 4 regions of the mica surface were examined to confirm the homogeneity of the structures throughout the sample. Mean particle heights were analyzed by averaging the measured values of 8 individual cross-sectional line scans from each image only when the particle structure was confirmed.

Electrophysiology

The field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 region of acute hippocampal slices derived from C57BL/6 mice (male, 4-5 weeks of age). The procedures for slice preparation and electrophysiological recording were described previously (Takamura *et al.* 2014). Briefly, 300- μ m thick transverse hippocampal slices were placed in a physiological chamber perfused with artificial cerebrospinal fluid (125 mM NaCl, 3.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 2.0 mM MgSO₄, 2.0 mM CaCl₂, and 20 mM glucose and aerated with a mixture of 95% O₂ and 5% CO₂) at a rate of 1 mL/min at 30°C. Shaffer collaterals/commissural bundle in the CA3 hippocampal subfield were stimulated using a bipolar stainless steel

wire electrode at 20-s intervals throughout the experiment. The fEPSPs were recorded from the stratum radiatum in the CA1 hippocampal subfield using a sharp glass electrode (2-6 Mohms, filled with 2 M NaCl). After fEPSP baseline became stabilized, slice was incubated with circulation of 10 ml α S sample (1 μ M) for 90 min on the experimental chamber without electric stimulation. fEPSP recording was restarted after incubation and confirmed baseline stability at least 20 min. LTP was induced by two train of tetanic stimulation delivered at 100 Hz for 1 s. The evoked potential was amplified ($\times 1000$), filtered (0.1-1000Hz), digitized (20 kHz), and stored in a computer for off-line analysis using the PowerLab system (AD Instruments, Colorado Springs, CO). LTP values were presented as the percentage of average fEPSPs slope relative to the mean value of the base line before tetanic stimulation.

NMR spectroscopy

The synthetic DNA encoding human α S was inserted into pOPTH plasmid. Sequencing of the inserted DNA was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The α S protein with an N-terminal His-tag (MAHHHHHH) was expressed in *E. coli* BL21(DE3) harboring the pOPTH plasmid. The cells were grown in M9 minimal medium supplemented with $^{15}\text{NH}_4\text{Cl}$ and ^{13}C -glucose. Purification of α S was done with a Ni-NTA agarose resin (QIAGEN), followed by further purification by gel-filtration on a Superdex 75 16/60 column (GE

Healthcare Bio-Sciences). Matrix-assisted laser desorption ionization time-of-flight mass (MALDI-TOF MS) analysis showed that His-tagged α S has no methionine at the N-terminus. MALDI-TOF MS analysis was performed on a Bruker Daltonics Autoflex-T1 mass spectrometer.

Stock solutions of Myr and RA (54 mM) were prepared by dissolution in dimethylsulfoxide. Aliquots of the phenolic solutions were mixed with a solution containing 41 μ M $^{13}\text{C}/^{15}\text{N}$ -synuclein, 10 mM HEPES (pH 7.4), 50 mM NaCl, 90 μ M NaN_3 , 90 μ M 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt and 10% D_2O . The polyphenol concentration was 0.41 mM so that the final α S : polyphenol concentration ratio was 1 : 10. An NMR sample of the α S alone was also prepared in the same manner without polyphenol. The NMR sample was incubated at 15°C for 6 days before NMR measurements. All NMR spectra were obtained at 15°C with a Bruker Avance 800 MHz spectrometer equipped with a cryoprobe (Bruker BioSpin, Rheinstetten, Germany). NMR data were processed with NMRPipe (Delaglio *et al.* 1995) and analyzed with NMRView (Johnson. 2004). The published backbone resonance assignments of α S (BMRB Entry 16300) were transferred to the data of His-tagged α S without polyphenol (Rao *et al.* 2009). The transferred assignments were confirmed by analyzing CBCANH, CBCA(CO)NH, HNCO and HN(CA)CO (Clubb *et al.* 1992; Grzesiek and Bax 1992a; Grzesiek and Bax 1992b; Grzesiek and Bax 1993).

NMR-based molecular modeling

The model of α S was obtained with the use of CS-ROSETTA and the chemical shift assignment data (Rao *et al.* 2009; Shen *et al.* 2008; Shen *et al.* 2009). CS-ROSETTA generated the ensemble of the disordered α S, and one of them was used to show the regions affected by the polyphenol-binding. Figure depicting the α S model were prepared with the program PyMOL (DeLano Scientific, New York, USA).

Statistical analysis

Dunnett multiple comparisons were used to determine statistical significance between cross-linked α S group versus other groups in LTP analysis. These tests were implemented within GraphPad Prism software (MDF, Tokyo, Japan). Significance was defined as $p < 0.05$.

Results

α S oligomerization

To determine whether the five phenolic compounds blocked formation of low n-order α S oligomers, we used PICUP, a photochemical cross-linking method that is rapid, efficient, requires no structural modification of α S, and accurately reveals the oligomerization state of α S (Bitan and Teplow 2004). Following cross-linking as

reported previously (Ono *et al.* 2012b), α S existed predominately as a mixture of monomers and oligomers of order 2-4, and higher order oligomers appear as smear bands (Fig. 2A). When 25 μ M Myr was mixed with α S at a peptide : compound ratio of 1 : 1, oligomerization was blocked, bands of tetramer and higher order oligomers disappeared, and intensity of trimer band was decreased (Fig. 2A). When 250 μ M of Myr was mixed with α S at a peptide : compound ratio of 1 : 10, oligomerization was blocked almost completely (Fig. 2A). When 25 (1 : 1) or 250 μ M (1 : 10) RA was mixed with α S at the same ratios as used above, comparable effects were observed on α S oligomerization (Fig. 2A).

With α S : FA at a 1 : 1 ratio, intensities of bands of tetramer and higher order oligomers were decreased (Fig. 2A). At a higher concentration of FA (α S : FA, 1 : 10), oligomerization was blocked almost completely. With α S : NDGA at a 1 : 1 ratio and 1 : 10 ratio, similar effects was observed on α S oligomerization (Fig. 2A). With α S : Cur at a 1 : 1 ratio, no inhibition of α S oligomerization was observed. With α S : Cur at a 1 : 10 ratio, tetramer band was disappeared, and intensity of trimer band was decreased.

The results indicated that Myr and RA had the stronger inhibitory effects on α S oligomerization, compared to FA, NDGA, and Cur. We confirmed dose dependence of inhibitions by Myr and RA (Fig. 2, C and D). The EC_{50} of Myr and RA for the α S oligomerization were 23.9 and 22.7 μ M, respectively.

A potential problem relates to the possibility that the inhibition of α S oligomerization could have resulted from an alternative compound, which may form from a possible side reaction of the inhibitor and the PICUP sensitizer. To evaluate this possibility, cross-linking reactions also were performed on GST, a positive control for the cross-linking chemistry (Fancy and Kodadek 1999). Uncross linked GST exhibited an intense monomer band and a relatively faint dimer band (Fig. 2B). Cross-linking produced an intense dimer band, which was expected because GST exists normally as a homodimer, as well as higher-order cross-linked species. No alterations in GST cross-linking were observed in the presence of Myr, FA, NDGA, Cur, or RA at either of the two protein : compound ratios tested, 1 : 1, 1 : 10. Thus, the significant inhibition of α S oligomerization is from a direct interaction with the phenolic compounds.

Size-exclusion chromatography

The waves of SEC were shown in Figure S1A. Reconstitution of the lyophilizates to a nominal concentration of 25 μ M in 20 mM Tris HCl buffer, pH 7.4, followed by SDS-PAGE analysis, showed that removal of reagents and phenolic compounds, lyophilization and reconstitution did not alter the oligomer composition of any of the peptide populations under study (Figure S1B).

α S assembly morphology

We used AFM and EM to determine the morphology of the small assemblies present following PICUP of α S with or without phenolic compounds. The height of uncross-linked α S was 0.53 ± 0.05 nm (Fig. 3 and Table 1) from AFM analysis. Following PICUP, the height of α S oligomers became 1.60 ± 0.24 nm. These morphologies are consistent with our previous findings (Ono *et al.* 2011; Ono *et al.* 2012b). When α S was cross-linked with Myr at a compound : peptide ratio of 1 : 2, the height of treated α S decreased to 0.58 ± 0.06 nm. When α S was cross-linked with RA at a compound : peptide ratio of 1 : 2, the height of treated α S was decreased to 0.57 ± 0.06 nm (Fig. 3 and Table 1). Similarly, when α S was cross-linked with NDGA, FA, or Cur at a compound : peptide ratio of 1 : 2, the height of treated α S decreased to 0.54 ± 0.06 nm, 0.57 ± 0.07 nm, or 0.60 ± 0.07 nm, respectively.

Similar data were obtained from EM analysis. The diameter of uncross-linked α S was 2.32 ± 0.17 nm. Following PICUP, the diameter of α S oligomers became 11.94 ± 1.51 nm. These morphologies are also consistent with our previous findings (Ono *et al.* 2012b). When α S was cross-linked with Myr, NDGA, FA, Cur, or RA at a compound : peptide ratio of 1 : 2, the diameters of treated α S decreased to 2.80 ± 0.34 nm, 2.70 ± 0.27 nm, 2.99 ± 0.38 nm, 3.15 ± 0.38 nm, or 2.67 ± 0.35 nm, respectively (Table 1).

α S secondary structure dynamics

The above oligomerization studies revealed effects of the phenolic compounds at the initial stages of peptide self-association. To examine whether the phenolic compounds altered the secondary structure of the α S, we undertook CD studies (Fig. 4). α S, incubated alone, produced initial spectra characteristic of statistical coils (Fig. 4A). The major feature of these spectra was a large magnitude minimum centered at ~ 198 nm. α S displayed substantial secondary structure changes between days 2-3 that were consistent with the previous study (Ono *et al.* 2012b). When α S were incubated with Myr and RA at a compound : peptide ratio of 1 : 2, no such transitions were observed (Fig. 4, B and C).

Secondary structures of α S assembly

We measured secondary structure of seeds, such as uncross-linked α S, cross-linked α S with or without Myr or RA, using CD studies. The seed of cross-linked α S without phenolic compounds produced spectrum characteristic of β -sheet, on the other hand, the seeds of uncross-linked α S and cross-linked α S with Myr or RA produced spectrum characteristic of statistical coils (Figure S2A).

Seeding activities of α S assemblies

α S fibril assembly proceeds along a nucleation-dependent polymerization process (Wood *et al.* 1999). To monitor the abilities of cross-linked α S with or without

phenolic compounds to exert fibril formation as seeds, we measured the time dependence of ThS fluorescence in seeded fibril formation experiments (Figure S2B). Uncross-linked α S displayed a quasisigmoidal process curve characterized by an ~ 6 hr lag time, an ~ 96 hr period of increasing ThS binding, and a binding plateau occurring after ~ 120 hr (Figure S2B). The unseeded reaction did not display initial fluorescence increase, within experimental error. Adding 10% cross-linked α S oligomers eliminated the lag period and produced a quasihyperbolic increase in fluorescence that reached maximal levels at ~ 48 hr, suggesting that cross-linked α S oligomers functioned as seeds. However, this seeding activity had disappeared in the cross-linked α S with Myr or RA (Figure S2B). Maximal ThS levels for the α S oligomers seeded reaction was reached in 72 hr, whereas those of seeded with uncross-linked α S, and cross-linked α S with Myr or RA reached in 120 hr.

Electrophysiology

To obtain an index of cross-linked α S-induced functional alteration of synaptic transmission, we analyzed LTP in the CA1 region of mouse hippocampal slices. Synaptic current strength was estimated from fEPSP slope (Fig. 5). The vehicle group indicated LTP by tetanus stimulation ($166 \pm 7.6\%$). Uncross-linked α S did not affect LTP ($177 \pm 15.5\%$). Cross-linked α S completely inhibited induction of LTP ($87 \pm 19.4\%$). In contrast, cross-linked α S treated with Myr and RA induced LTP

comparable with that in the vehicle ($150 \pm 15.6\%$ and $155 \pm 18.1\%$, respectively). Fig. 5 shows differences in LTP induction among the five treatment groups. There was a significant group effect on %fEPSP slope in the cross-linked α S group was significantly lower than those in the other four groups, indicating that cross-linked α S induced LTP suppression, but cross-linked α S treated with Myr and RA did not.

NMR studies

In order to study the interaction between the phenolic compounds and α S, we utilized NMR spectroscopy, a widely accepted method to obtain atomic level aspects of protein structure and ligand binding. Fig. 6 shows the overlaid ^1H - ^{15}N HSQC spectra of the α S alone and the α S containing the phenolic compounds at 1 : 10 molar ratio (α S : polyphenol). The ^1H - ^{15}N HSQC spectra of α S are typical of an unstructured protein with limited resonance dispersion in the proton dimension (Wu et al. 2008).

Myr causes the reduction in the signal intensities due to the broadening of NMR resonances (Fig. 6A). The broadening results from the interactions between Myr and α S. Severe broadening was observed only within the first nine residues of α S, indicating that the N-terminal region was involved in the Myr-binding (Fig. 6A). On the other hand, neither chemical shift changes nor line broadening was observed upon RA addition, suggesting that RA did not bind to monomeric α S (Fig. 6B).

Discussion

We previously reported that several antioxidants including the phenolic compounds, Myr, FA, NDGA, Cur, and RA, had the inhibitory effects on α S fibrillization and α S fibril-destabilizing effect (Ono *et al.* 2003). In this study, we revealed that all five phenolic compounds had dose-dependently inhibitory effects on α S oligomerization, using the PICUP studies. Using EM and AFM analysis, the diameters and the heights of cross-linked α S treated with phenolic compounds were smaller than cross-linked α S oligomers. The CD studies unraveled that Myr and RA stabilized α S populations comprising mostly random coil and inhibited statistical coils $\rightarrow$ β -sheet conversion. In ThS assay, cross-linked α S treated with Myr and RA lost seeding activities. Taken together, we revealed that the abilities of Myr and RA to inhibit α S oligomerization and secondary structure conversion. To unravel chemical and neurophysiological basis for these effects, we performed LTP experiment and NMR analysis. Myr and RA decreased synaptic toxicities induced by α S oligomers on LTP assay of hippocampal slices. NMR showed the direct binding of Myr to the first nine residues of the N-terminal region of the monomeric α S protein, on the other hand, the direct binding of RA to the α S monomer was not detected.

What is the mechanism underlying the inhibitory effects of the phenolic compounds on α S oligomerization? The binding of Myr to the first nine residues of the N-terminus of α S in the NMR experiment might contribute to inhibition of α S

oligomerization. The sequence of α S can be divided into three domains: the N-terminal domain, the central fragment, also known as NAC (non-amyloid β component) region, and the C-terminal region. NAC region (residues 61-95) was reported to represent the critical determinant of the oligomerization and the fibrillation process of α S (Hejjoui *et al.* 2012). The C-terminal region (residues 96-140) is highly disordered and negatively charged, and it was shown that negatively charged side chains located in C-terminal region of α S acted to retard fibril formation by thioflavin T (ThT) binding assay (Izawa *et al.* 2012). Similar to Myr, a small molecular tweezer, CLR01, was recently reported to bind selectively to Lys side chains at the N-terminal region of α S and prevent its aggregation by electron-capture dissociation mass-spectrometry and PICUP study (Acharya *et al.* 2014). Using ThT fluorescence and EM, it is shown that in the presence of CLR01, α S did not form amyloid fibrils, and demonstrated that CLR01 inhibited α S-mediated toxicity in cell cultures and zebrafish embryos (Prabhudesai *et al.* 2012). The data of fluorescence and mass-spectrometric analysis suggested that CLR01 kept α S monomeric by increasing its reconfiguration rate (Acharya *et al.* 2014; Prabhudesai *et al.* 2012). It was shown that the polyphenol 3,4-dihydroxyphenylacetic acid (DOPAC) bound to the N-terminal region of α S and inhibited fibrillation of α S binding non-covalently (Zhou *et al.* 2009). By deleting residues 2-11 in the N-terminal, α S aggregation was delayed and cellular membrane permeabilization of α S monomer and oligomer could be abolished (Lorenzen *et al.*

2014a). It was shown that the N-terminal deletions in α S dramatically reduced toxicity towards yeast (Vamvaca *et al.* 2009). On the other hand, an NMR analysis revealed that EGCG noncovalently bound to C-terminal region of the monomeric α S (D119, S129, E130, and D135), and redirected α S into unstructured, off-pathway α S oligomers (Ehrnhoefer *et al.* 2008). EGCG did not affect oligomer size distribution or secondary structure, and rather, immobilized the C-terminal region and moderately reduced the degree of binding of oligomers to membrane (Lorenzen *et al.* 2014b). Based on the results of these studies (Acharya *et al.* 2014; Prabhudesai *et al.* 2012; Zhou *et al.* 2009; Lorenzen *et al.* 2014a; Vamvaca *et al.* 2009) and our NMR study, the binding region of α S with phenolic compounds might make equilibrium shift of α S aggregation capacities among the N-terminal, NAC, and the C-terminal region toward the suppression of aggregation. Interestingly, the anti-oligomerization effects of phenolic compounds on α S are similar to those on A β in our previous study (Ono *et al.* 2012a). In our previous NMR study with A β , Myr showed binding to monomeric A β at Arg-5, Ser-8, Gly-9, His-13, Lys-16, Asp-23, and Ile-31 (Ono *et al.* 2012a). There were no common amino acid sequences in Myr binding sites between A β and α S. Unlike the result of Myr, we found no direct binding of RA to monomeric α S in NMR study. In contrast, a previous NMR study reported the interaction between α S and RA, indicating that residues 3-18 and 38-51 of α S acted as noncovalent binding sites for RA (Rao *et al.* 2008). The discrepancy between the previous study (Rao *et al.* 2008) and the present study might

be related to the lower concentration of α S used in the present study.

The ability of Myr and RA to block formation of low-order α S oligomers in our study is valuable, because low-order α S oligomers were thought to be the proximate neurotoxins in α -synucleinopathies from the results of several *in vitro* and *in vivo* studies (Outeiro *et al.* 2008; Paleologou *et al.* 2009; Tsigelny *et al.* 2008). Visualization of α S oligomerization in living cells using bimolecular fluorescence complementation revealed that formation of oligomeric α S species was a central step toward cytotoxicity, which can be targeted through the activity of molecular chaperones, such as heat shock protein 70 (Outeiro *et al.* 2008). Consistent with this result, toxicity was seen without heavily aggregated α S in the experiment of dopaminergic and non-dopaminergic neurons, and it has been suggested that soluble species mediate toxicity (Xu *et al.* 2002). We took into consideration these cumulative evidences, and performed LTP assay of hippocampal slices to evaluate synaptic toxicities induced by α S oligomers. LTP has been widely used as a neurophysiological model of activity-dependent synaptic plasticity, and are considered as important neurophysiological models of learning and memory (Martin *et al.* 2000). It has been reported that human A β oligomers inhibit hippocampal LTP *in vitro* and *in vivo* in rats (Townsend *et al.* 2006). However, knowledge about the effects of α S oligomers on synaptic plasticity is currently limited. It was reported that the exposure to α S oligomers impaired LTP through NMDA receptor activation, triggering enhanced

contribution of calcium-permeable AMPA receptors in rat hippocampus slices (Diógenes *et al.* 2012). The application of extracellular α S oligomers was reported to induce LTP suppression in hippocampal neurons via a calcineurin-dependent mechanism (Martin *et al.* 2012). Our work was consistent with previous studies (Martin *et al.* 2012; Diógenes *et al.* 2012), where α S oligomers, not monomers, suppressed LTP in the hippocampal CA1 subfield, suggesting that memory formation is disturbed by α S oligomers. In contrast, cross-linked α S treated with Myr or RA partly cured LTP suppression. This result suggests that Myr and RA have preventive effects on α S oligomer-induced synaptic dysfunction by interfering with α S oligomerization. In recent studies, Cur showed effectiveness on motor activity, lifespan, oxidative stress, and apoptosis in the transgenic *Drosophila* model of Parkinson's disease (Siddique *et al.* 2013; Siddique *et al.* 2014). *In vivo* experiments with animal models of α -synucleinopathies should be conducted to elucidate the effectiveness of phenolic compounds on α -synucleinopathies.

To develop the therapies for α -synucleinopathies by phenolic compounds, we need to overcome several issues. The first issue is low bioavailability of phenolic compounds. Cur displayed low oral bioavailability, poor water-solubility, short biological half-life, and lower permeability through the brain blood barrier (BBB) (Anand *et al.* 2007). According to the data of clinical trial of Cur, bioavailability of Cur was not high; the range for serum concentration was between $0.51 \pm 0.11 \mu\text{M}$ at a dose of 4000 mg/day

and $1.77 \pm 1.87 \mu\text{M}$ at a dose of 8000 mg/day (Cheng *et al.* 2001). A dose escalation study showed the safety of Cur intake in healthy volunteers, thus they took oral Cur ranged from 500 to 12000 mg and serious adverse events were not reported (Lao *et al.* 2006). To our best knowledge, there have been no reports about the orally-ingested Cur concentrations in cerebrospinal fluid. We have insufficient knowledge about bioavailability of Myr and RA, and we need to obtain further information about bioavailability of phenolic compounds. The next issue is the possibility that phenolic compounds may affect physiological functions of αS at synapses. Further experiments are necessary to elucidate whether extracellular phenolic compounds or intracellular phenolic compounds taken into the cell alter the synaptic functions. Specific targeting of extracellular αS has an additional advantage as a therapeutic strategy, as this approach will not interfere with the normal function of intracellular αS . Although the most of αS , a neuronal presynaptic protein, exists intracellularly, the secreted extracellular αS , particularly in oligomerized forms, was reported to play important roles in major pathological changes of α -synucleinopathies: deposition and spreading of aggregates, neuroinflammation, and neurodegeneration (Lee *et al.* 2014). We speculate that anti-oligomerization effects of phenolic compounds, such as Myr and RA, might reduce the formation of extracellular αS oligomers as well as intracellular αS oligomers, resulting in favorable effects on α -synucleinopathies.

In conclusion, our data established that the phenolic compounds inhibit

oligomerization and statistical coils $\rightarrow$ β -sheet conversion of α S through different α S binding, and reduce α S oligomer-induced synaptic toxicity. Although the exact *in vivo* mechanisms underlying the benefits of polyphenols remain to be established, the present data, coupled with previously reported antioxidant and neuroprotective effects, suggest that phenolic compounds would be considerable candidates of disease modifying therapies for α -synucleinopathies.

Acknowledgments and conflict of interest disclosure

This work was supported by a grant for the Hokuriku Innovation Cluster for Health Science (M.Y.) and a Grant-in-Aid for Scientific Research (C) (26461266) (K.O.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, a grant for the Amyloidosis Research Committee (M.Y.) from the Ministry of Health, Labour, and Welfare, Japan, a grant from Takeda Science Foundation (K.O.), and a grant from Life Science Foundation of Japan (K.O.). No potential conflicts of interest were disclosed.

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Figure Legends

Fig. 1. Chemical structures of Myr, FA, NDGA, Cur, and RA.

Fig. 2. α S and GST oligomerizations. PICUP, followed by SDS-PAGE and silver staining, was used to determine the effects of 25 and 250 μ M Myr, FA, NDGA, Cur, or RA on oligomerization of α S (A) or GST (B). Consequently, we showed concentration dependent inhibitory effects of α S oligomerization of Myr (C) or RA (D). +, with cross-linking; -, without cross-linking. The gel is representative of each of three independent experiments.

Fig. 3. α S assembly morphology. AFM was performed on 25 μ M uncross-linked (A) and cross-linked (B-G) α S without (B) or with 50 μ M Myr (C), FA (D), NDGA (E), Cur (F), or RA (G). EM was performed on 25 μ M uncross-linked (H) and cross-linked (I-N) α S without (I) or with 50 μ M Myr (J), FA (K), NDGA (L), Cur (M), or RA (N). Scale bars indicate 100 nm.

Fig. 4. α S secondary structure dynamics. CD was used to monitor peptide assembly. 25 μ M α S (A-C) were incubated at 37°C for 5 days in 20 mM Tris-HCl buffer, pH 7.4, in buffer alone (A) or in the presence of 50 μ M Myr (B) or RA (C). Spectra were acquired immediately at the start of the incubation period, day 0 ($\circ$), and after days 1

(●), 2 (□), 3 (■), 4 (△), and 5 (▲). The spectra presented at each time are representative of those obtained during each of three independent experiments.

Fig. 5. Effects of Myr or RA on cross-linked α S-induced alterations of LTP in the hippocampal slices. (A) Synaptic toxicity. Typical fEPSP waveforms of pre- (black lines) and post-tetanic (dashed red lines) stimulation in each test group. Thirty waveforms were averaged. (B) Time course of %fEPSPs slope. Arrows indicate tetanus stimulation (100 Hz, 1 s, 2 train). (C) Values are shown as the percentage of fEPSP slope relative to the baseline and presented as mean $\pm$ S.E. Significant difference between cross-linked α S versus other experimental group, along with their associated *p*-values, where * signifies *p* < 0.05 and ** signifies *p* < 0.01

Fig. 6. Analysis of interaction between α S and polyphenols. (A, B) ^1H - ^{15}N HSQC spectra of α S in the absence of (black contour) and in the presence of (red contour) polyphenols. (A) Myr and (B) RA. Residues with intensity ratios ($I_{\text{bound}}/I_{\text{free}}$) < 0.15 are labeled. An unassigned resonance is indicated by asterisk. (C, D) Model representation of α S that show binding locations with polyphenols. The red-colored regions are the residues that showed the peak broadening in the presence of polyphenols. (C) Myr and (D) RA.

Table 1. Morphological analysis of α S assemblies

Assembly	Diameter ^a	Height ^b
Uncross-linked α S	2.32 ± 0.17 (30)	0.53 ± 0.05 (30)
Cross-linked α S	11.94 ± 1.51 (30)	1.60 ± 0.24 (30)
Cross-linked α S with Myr	2.80 ± 0.34 (30)	0.58 ± 0.06 (30)
Cross-linked α S with NDGA	2.70 ± 0.27 (30)	0.54 ± 0.06 (30)
Cross-linked α S with FA	2.99 ± 0.38 (30)	0.57 ± 0.07 (30)
Cross-linked α S with Cur	3.15 ± 0.38 (30)	0.60 ± 0.07 (30)
Cross-linked α S with RA	2.67 ± 0.35 (30)	0.57 ± 0.06 (30)

^aMean diameter $\pm$ SE, in nanometers, is listed for (n) α S assemblies visualized by EM.

^bMean diameter $\pm$ SE, in nanometers, is listed for (n) α S assemblies visualized by AFM.

Supporting information

Figure S1. Size exclusion chromatography (SEC) of α S assemblies. SEC was performed on 25 μ M cross-linked α S without phenolic compound (A), or with 50 μ M Myr (B) or RA (C) for removing reagents. Fractions were lyophilized immediately after collection. Lyophilization and reconstitution did not alter the oligomer composition of any of the peptide populations under study (D). Lanes 1-2 showed pre-SEC state of uncross-linked and cross-linked α S. Lanes 3, 5, and 7 showed pre-SEC state, and Lanes 4, 6, and 8 showed post-SEC state of cross-linked α S without or with Myr or RA, respectively.

Figure S2. The secondary structures and seeding activities of α S assemblies. CD was used to measure the secondary structure of 25 μ M uncross-linked α S ($\circ$; UnXL), cross-linked α S without ($\bullet$; XL), or with 50 μ M Myr ($\square$; XL+Myr) or RA ($\times$; XL+RA) (A). 10% (v/v) uncross-linked ($\circ$; UnXL), or 25 μ M cross-linked α S without ($\bullet$; 10% XL) or with Myr ($\square$; 10% XL+Myr) or RA ($\times$; 10% XL+RA) was added to uncross-linked α S, which then was incubated for 24-168 hr at 37°C in 20 mM Tris buffer, pH 7.4. Aliquots were assayed periodically using ThS. Binding is expressed as mean fluorescence (in arbitrary fluorescence units [FU]) $\pm$ SE. ThS fluorescence were measured at 0, 1, 2, 6, 24, 48, 72, 96, 120, 144, and 168 hrs. Data was obtained in three independent experiments (B).

Fig. 1

Myr

FA

NDGA

Cur

RA

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Figure S1

Figure S2

A

B