Cytoprotective effect of chlorogenic acid against α-synuclein-related toxicity in catecholaminergic PC12 cells

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Parkinson's disease is a major neurodegenerative disease involving the selective degeneration of dopaminergic neurons and α-synuclein containing Lewy bodies formation in the substantia nigra. Although α-synuclein is a key molecule for both dopaminergic neuron death and the formation of inclusion bodies, the mechanism of α-synuclein induction of Parkinson's disease-related pathogenesis is not understood. In the present study, we found that the interaction between dopamine and α-synuclein requires the oxidation of dopamine. Furthermore, we examined the protective effect of chlorogenic acid, a major polyphenol contained in coffee, against α-syn and dopamine-related toxicity. Chlorogenic acid inhibits several DA/α-synuclein-related phenomenon, including the oxidation of dopamine, the interaction of oxidized dopamine with α-synuclein, and the oligomerization of α-synuclein under dopamine existing conditions *in vitro*. Finally, we showed that the cytoprotective effect against α-synuclein-related toxicity in PC12 cells that can be controlled by the Tet-Off system. Although the induction of α-synuclein in catecholaminergic PC12 cells causes a decrease in cell viability, chlorogenic acid rescued this cytotoxicity significantly in a dose dependent manner. These results suggest that the interaction of oxidized DA with α-synuclein may be a novel therapeutic target for Parkinson's disease, and polyphenols, including chlorogenic acid, are candidates as protective and preventive agents for Parkinson's disease onset.

Key Words: chlorogenic acid, polyphenol, α-synuclein, Parkinson's disease, dopamine

Parkinson's disease (PD) is a neuropathological disorder involving the selective degeneration of dopaminergic neurons and α-synuclein (α-syn) containing Lewy body (LB) formation in the substantia nigra, and the subsequent loss of their terminals in the striatum.[1,2] The ensuing loss of dopamine (DA) causes most of the debilitating motor disturbances associated with PD.

Abundant risk factors, including genetic and environmental factors, that can induce and/or enhance PD onset have been reported.[3,4] The molecule, α-syn, is an important molecule associated with the pathogenesis of familial and sporadic PD. α-syn is a major component of LBs, and a pathogenic molecule that can induce familial PD, PARK1 and PARK4.[3] In the PARK4 family, multiplication of the α-*syn* gene, which results in the overexpression of α-syn, induces the loss of dopaminergic neurons and typical parkinsonism.[5–7] Furthermore, the overexpression of α-syn, including pathogenic mutations found in the PARK1 family, have been reported to undergo DA-related cell death.[8] Previously, we reported that the overexpression of α-syn in catecholaminergic PC12 cells shows DA-related vulnerability to several toxicities

including ER stress.[9] However, it is not understood why dopaminergic neurons specifically are disturbed by the overexpression of α-syn.

The regions of α-syn are classified to three major parts: an *N*-terminal region (residue 1–60) that encodes repetitive sequence including KTKEGV motif, a middle part (residue 61–95) that encodes an NAC domain forming the amyloidogenic core of α-syn, and a *C*-terminal sequence (residue 94–140) with a negatively charged region.[10–12] Oligomerization of α-syn is believed to be an important step for pathogenic toxicity, and several post-translational modifications such as phosphorylation,[13,14] nitration or oxidation at tyrosine residue,[15] glycosylation,[16] and lipid-modified form[17] have been reported as initiators for oligomerization and/or aggregation. However, these modifications are not phenomena specific to DA neurons.

It has been reported that DA can be converted to dopaminequinone (DAQ) following oxidation, and its oxidized form is believed to be toxic to cells.[18,19] In general, quinone attacks cysteine residues in proteins, and its oxidative modification of proteins may cause dysfunction. However, there is no cysteine residue in human α-syn. Therefore, it is unclear how α-syn induces cytotoxicity in the dopaminergic cells. A recent paper suggests that oxidized catechol interacts with the *C*-terminal sequence of the α-syn, and its interaction determines the oligomerization of α-syn.[20]

Antioxidants and/or quinone scavengers can inhibit the toxic effects of oxidized α-syn,[19] and numerous compounds including polyphenols have been examined as to their protective effects against PD-related cytotoxicity.[21,22] The results of a large scale cohort study suggest that coffee intake is inversely associated with the onset of PD, and one of the major components of coffee, caffeine, may play an important role on this prevention.[23,24] Although the effects of chlorogenic acid (CGA), a major component of coffee, have not been well investigated, an inhibitory effect of CGA on the oligomerization of α-syn *in vitro* has been reported, as well as other polyphenols.[25,26] In addition, since free radical scavenging effects of CGA have been also reported,[27,28] it seems to be useful to prevent PD onset. Therefore, we investigated the protective effects of CGA on DA and α-syn-related cytotoxicity in catecholaminergic PC12 cells.

Materials and Methods

Chemicals and antibodies. Nerve growth factor (NGF) was purchased from Invitrogen (Carlsbad, CA). Tet System Approved

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fetal bovine serum (FBS) and doxycycline (Dox) were obtained from Clontech (Palo Alto, CA). Mouse monoclonal antibody against human α-syn and rabbit polyclonal antibody against β-actin were purchased from BD Transduction laboratory (Lexington, KY) and Cell Signaling Technology (Beverly, MA), respectively. HRP-linked anti-mouse IgG and anti-rabbit IgG were from Amersham Biosciences (Buckinghamshire, UK). Cysteine, L-DOPA, DA, and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrasodium bromide (MTT) were obtained from Wako (Osaka, Japan). α-Methyltyrosine (aMT) was purchased from Pfaltz & Bauer (Waterbury, CT). Chlorogenic acid was obtained from MP Biomedicals, LLC (Irvine, CA). PBA was purchased from Sigma. ^{3}H-DA was prepared from Perkin Elmer (Norwalk, CT). Recombinant α-syn was obtained from Bio Mol (Plymouth Meeting, PA)

Preparation and culture of α-syn expressing PC12 cells.
α-syn expressing PC12 cells controlled Tet-Off system (Clontech) (PC12-α-syn-Tet Off) were prepared as described previously.[9] During the amplification of PC12-α-syn-Tet Off cells, cells were maintained at 37.0°C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium, supplemented with 5% FBS, 10,000 unit/ml penicillin (PS) and 100 mg/ml streptomycin (SM) and 2 ng/ml Dox. In order to induce α-syn expression, the culture medium was changed to Dox-free medium (DMEM/F12, 2.5% Tet System Approved FBS, and PS/SM). For induction of neural differentiation, 50 ng/ml NGF was added during culture. The culture medium was exchanged for fresh medium in order to delete Dox completely. For the present experiments, the cells were cultured for 3–7 days.

Cell viability assay (MTT assay). Cell viability was measured using the MTT assay following the protocol described previously with some modifications.[24] For the MTT assay, 1×10^3 cells/well were seeded in 96-well plates, cultured for 3 or 7 days, and then incubated with MTT for 2 h at 37°C. After adding 100 μl of 0.05 N HCl in 2-propanol and mixing thoroughly to dissolve the dark blue crystal, the MTT reduction was measured with a microplate reader (Bio-Rad; wavelength of 570 nm). The data were presented as percent post-treatment recovery (percent live cells) where the absorbance from the control, non-treated cells was defined as 100% live cells.

For analyzing the role of catecholamine on α-syn cytotoxicity in PC12 cells, PC12 cells were cultured with or without aMT, a specific inhibitor of tyrosine hydroxylase. Similarly, in order to investigate the protective effect of cysteine and CGA on α-syn toxicity in PC12 cells, each compound was added to the culture medium, and cell viability was measured by MTT assay.

SDS-PAGE and western blot analyses. For SDS-PAGE and immunoblot analyses, cells were harvested from 6-well culture plates, and lysed in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1 mM PMSF, 2 mM EDTA). Aliquots (20 μg) were separated by size on 12% polyacrylamide gels. SDS-PAGE was performed following the usual Laemmli's protocol and western blotting was performed as described previously.[29]

α-syn oligomerization analysis. For *in vitro* aggregation analyses, a mixture of 20 μM α-syn, 10 mM Tris-HCl, pH 7.8, and 1 mM DA or L-DOPA was incubated at 30°C for 12 h. To investigate whether CGA can inhibit the oligomerization of α-syn, a comparison study with or without CGA was performed. Reaction mixture was lysed in SDS-sample buffer, and subjected to SDS-PAGE. Soluble oligomer of α-syn was detected using a silver staining kit (Wako, Osaka, Japan) according to the manufacturer's protocol.

Measurement of DA/DOPA oxidative products by spectrophotometer. Catecholamine is known to be oxidized into catecholamine-quinone in alkaline pH. DA and DOPA are oxidized into DAQ and DOPA-quinone (DOPAQ). These are auto-oxidized into dopaminochrome (DAch)/dopachrome (DOPAch) and melanins, which are detectable by measuring the absorbance at 490 nm. DA (100 μM) or DOPA (100 μM) was incubated in 10 mM Tris-

HCl, pH 7.8 buffer with or without 1 mM of CGA for 0–20 h. Equal amounts of the reactive products were plated into 96 well micro plates and the absorbance was measured by a microplate reader (Perkin Elmer; 490 nm) to quantify the amount of the DA/DOPA oxidative products. To examine the inhibitory effect of cysteine and CGA on the formation of DA/DOPA oxidation, each compound was added to the reaction mixture.

Direct binding of ^{3}H-DA and α-syn. α-syn (2 nM) and ^{3}H-DA (10 nM) were incubated in 200 μl of 10 mM Tris-HCl buffer of various pH (range, 6.6–8.2) at 30°C for 30 min. After incubation, the reaction was terminated by adding 200 μl of activated charcoal suspension. After 30 min of absorption, α-syn bound ^{3}H-DA and unbound ^{3}H-DA (absorbed by the charcoal) were separated by centrifugation for 3 min at $1,000 \times g$. Aliquots (350 μl) of the supernatant were transferred to another tube and 3 ml of scintillation cocktail (Perkin Elmer) was added. α-syn bound ^{3}H-DA in the supernatant was analyzed by liquid scintillation counter. α-syn (2 nM) and ^{3}H-DA (10 nM) were incubated in 10 mM Tris-HCl, pH 7.8 along with an increasing concentration of CGA (0–10 μM) for 30 min at 37°C, and the α-syn bound ^{3}H-DA was measured by the same method described above.

Aminophenylboronate agarose (PBA) isolation of DAQ-modified α-syn. Analyses for the binding between α-syn and DAQ using PBA were carried out following the protocol described by Bisaglia[30] with some modifications. Briefly, α-syn was dissolved at a final concentration of 0.5 μg/ml in 500 μl of 10 mM Tris-HCl, pH 8.2 in the presence of 1 mM DA. PBA suspension (100 μl) was added and mixtures were incubated at 4°C for 12 h. After 3×30 min washes with 14 ml each of 1 mM Tris buffer (pH 8.6), α-syn bound to PBA was eluted with 2×500 μl of 50 mM glycine. Finally, samples were analyzed by SDS-PAGE and western blotting for α-syn. As a control, we performed identical experiments in the absence of DA.

Results

Overexpression of human α-syn causes a decrease of cell viability in PC12 cells. Withdrawal of Dox induced a marked increase of α-syn expression (Fig. 1A) and gradually decreased cell viability (72.5% on day 3, 56.6% on day 7 vs CTL, respectively) in PC12 cells (Fig. 1B). aMT, a specific inhibitor of tyrosine hydroxylase, was used to evaluate the association between α-syn toxicity in PC12 cells and endogenous catecholamine metabolism. Simultaneous administration of aMT (100 μM) abolished the α-syn-related cytotoxicity significantly (81.8% on day 3, 99.2% on day 7, vs CTL, respectively).

DA and DOPA enhance the oligomerization of α-syn *in vitro*. In order to investigate the effects of DA and DOPA on α-syn oligomerization, a simple experiment using recombinant human α-syn protein and DA or DOPA was performed *in vitro*. Under alkaline conditions, DA and DOPA induced a soluble oligomer of α-syn (Fig. 2).

DA is converted to the oxidized forms DAQ and/or DAch and binds to α-syn under alkaline condition. Since DA and DOPA can be converted to the oxidized forms, DAQ/DOPAQ or DAch/DOPAch, under alkaline condition, we quantified the oxidized forms of DA and DOPA under several pH conditions. Both DA and DOPA were converted to oxidized forms, especially under alkaline conditions (Fig. 3A).

We also performed DA/α-syn binding assays under several pH conditions using ^{3}H-labeled DA. Higher binding activity between DA (or its metabolites) and α-syn was observed under alkaline conditions where DA can be converted to the oxidized form (Fig. 3B).

We hypothesized that the oxidized form of DA may bind to α-syn directly, therefore, we examined the binding between DAQ and α-syn using PBA following the protocol described previously.[30] *In vitro* experiments demonstrated the binding activity
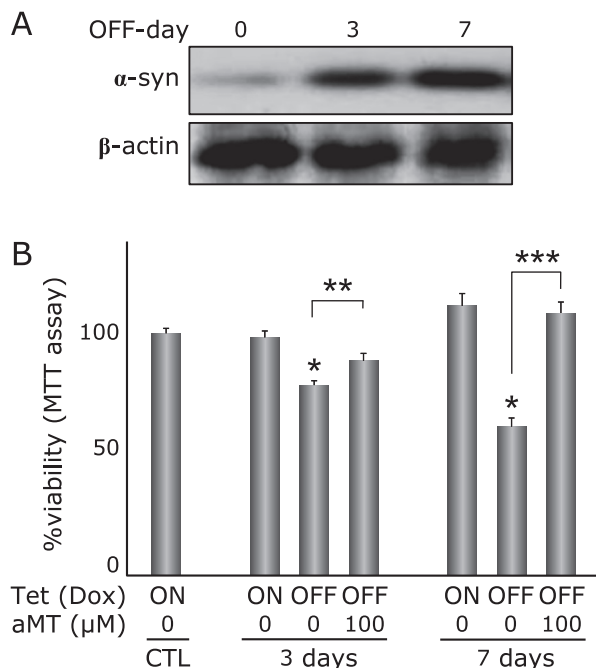
Fig. 1. α-syn expression induces catecholamine dependent cell death in PC12 cells. (A) Withdrawal of Dox for 3 or 7 days induces α-syn expression. Day 0 is the α-syn suppressed condition (2 μg/ml Dox). Top panel shows inducible expression of α-syn, and the bottom panel shows β-actin as a loading standard. (B) Withdrawal of Dox for 3 or 7 days decreases cell viability (MTT assay) to 72.5 and 56.6%, respectively. Simultaneous treatment with aMT (100 μM) abolishes the toxicity by α-syn and upregulates those viabilities to 81.8 and 99.2%, respectively, suggesting that α-syn-related toxicity is associated with catecholamine metabolism. Each column is an average of 6 independent experiments, and the bar means SE. Asterisk means statistically significance *p<0.01 vs CTL, **p<0.05, ***p<0.01, respectively (ANOVA).
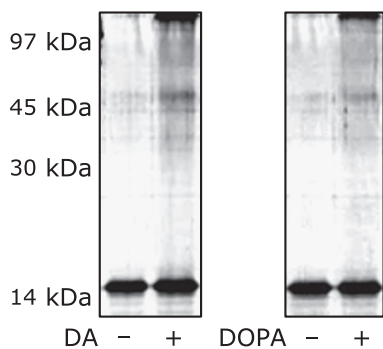

Fig. 2. DA and DOPA mediate formation of α-syn soluble oligomers. α-syn was incubated alone or in the presence of DA (100 μM) or DOPA (100 μM) in Tris-HCl, pH 7.8 at 37°C for 24 h. Co-incubation of α-syn with DA or DOPA enhances α-syn oligomerization *in vitro*. The reaction mixture was separated by SDS-PAGE, and α-syn monomer and oligomer are detected by silver staining.

between DAQ and α-syn (Fig. 3C). Moreover, we investigated whether intracellular α-syn binds to endogenous DA using cell lysate prepared from PC12-α-syn-Tet Off conditions. Under Tet Off conditions, induced α-syn was co-precipitated with PBA/DAQ (Fig. 3D). On the other hand, in the experiment using extract from cells that endogenous metabolism of catecholamine was inhibited by aMT administration, little α-syn was precipitated compared with results using cells capable of catecholamine metabolism (Fig. 3D).

The quinone scavenger cysteine suppresses DAQ/DAch formation, α-syn oligomerization, and α-syn-related cytotoxicity in PC12 cells. In the present study, results suggest that the oxidized form of DA, DAQ, may play an important role on α-syn-related cytotoxicity. In order to confirm this hypothesis, we examined the protective effects of the quinone scavenger cysteine on α-syn-related cytotoxicity in PC12 cells. Co-incubation of cysteine decreased DAQ/DAch formation and α-syn oligomerization under DA existing condition in alkaline pH (Fig. 3 E and F). Furthermore, we confirmed the cytoprotective effects of cysteine against DA/α-syn-related toxicity in PC12 cells. Simultaneous treatment with cysteine rescued the cells from DA/α-syn induced toxicity significantly (Fig. 3G).

CGA inhibits DA-related oligomerization of α-syn and rescues the cells from α-syn cytotoxicity. We investigated whether the presence of some antioxidative substance could prevent DA oxidization and subsequent α-syn oligomerization. Since CGA has been reported to have an inhibitory effect against α-syn oligomerization,[25] we examined the effects of CGA on α-syn oligomerization and DA/α-syn-related toxicity.

We investigated the effects of CGA on DA and/or DOPA oxidation under alkaline condition. CGA dramatically inhibited DAQ/DAchrome formation in alkaline pH buffer (Fig. 4A). We examined the inhibitory effects of CGA on α-syn oligomerization under DA existing and alkaline conditions. CGA also inhibited direct binding of ^{3}H-DA (or its oxidized metabolites) and α-syn in a dose dependent manner (Fig. 4B). Co-incubation of CGA with DA /DOPA and α-syn showed suppressive effects of CGA against both DA- and DOPA-related soluble oligomer formation of α-syn (Fig. 4C).

Finally, we examined the protective effects of CGA on PC12-α-syn-Tet Off cells. Simultaneous and continuous treatment of CGA rescued the PC12 cells against α-syn-related cytotoxicity in a dose dependent manner (Fig. 5).

Discussion

α-syn is a key molecule in PD pathogenesis that relates to not only familial PD, but also LB formation in the brain from sporadic PD.[1–4] Overexpression of α-syn in the PARK4 family resulted in a typical parkinsonian phenotype and cell loss of dopaminergic neurons, as well as usual sporadic PD.[5–7] However, it has not been well understood why high expression levels of α-syn induces selective cell death of the dopaminergic neurons. Some reports suggest that α-syn shows cytotoxic character only in the dopaminegic cells, but not in the non-dopaminergic cells.[8,9,31] Conway reported that some compounds with the catechol structure, including DA and DOPA, form adducts with α-syn,[32] and its unstable protofibril may one of the causes for α-syn-related toxicity. Indeed, several reports suggest that DA and/or catecholamine-associated materials interact with α-syn, especially with C-terminal sequence in α-syn. Moreover, the association between DA/DAQ and PD-related molecules, including parkin,[33] tyrosine hydroxylase,[34] dopamine transporter,[35] α-syn,[36,37] ubiquitin,[28] has been reported. These results suggest that the functional interaction between DA (and/or related materials) and α-syn should be considered as important in the pathogenesis in PD.

We previously established PC12-α-syn-Tet Off cells that can be controlled in the expression level of α-syn by Dox withdrawal from the culture medium.[9] In this cell line, catecholamine metabolism can be also inhibited by aMT. Therefore, it may be a useful model for the investigation of the pathogenic interaction between DA and α-syn. Using this cell line, we confirmed catecholamine metabolism-related cytotoxicity of α-syn.
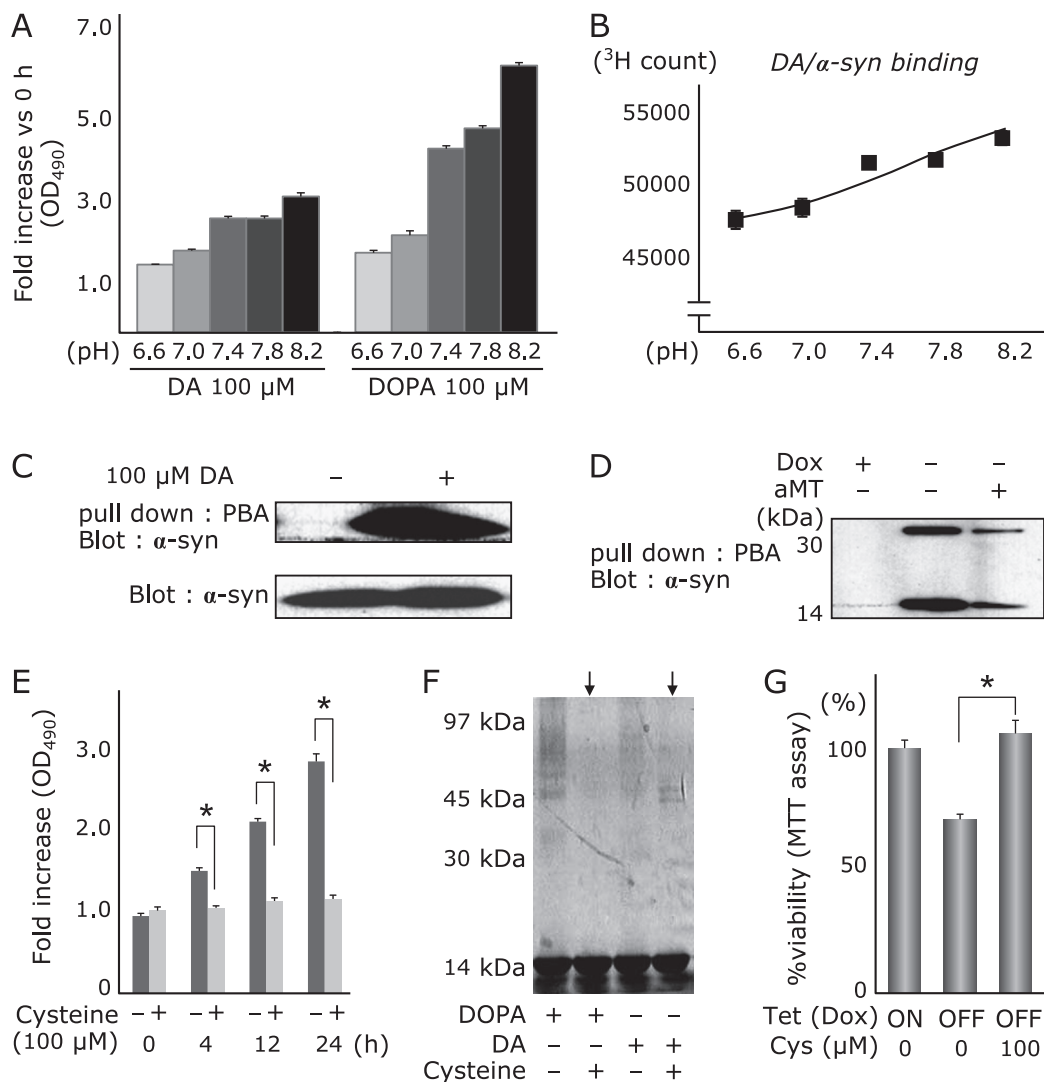
Fig. 3. Oxidized DA or DOPA is required for the oligomerization of α-syn. (A) pH dependent oxidation of DA and DOPA. DA (100 μM) or DOPA (100 μM) was incubated in increasing pH Tris-HCl buffer (range, 6.6–8.2) at 37°C for 0–20 h, and 100 μl of the reaction mixtures were analyzed by a spectrometer at 490 nm. Alkaline condition dependent DA/DOPA oxidation is shown. (B) α-syn/DA binding assay using ^{3}H-labeled DA. pH dependent interaction between α-syn and DA (its oxidative DA; DAQ) is demonstrated. DA (or its metabolites) and α-syn interact with each other in the alkaline condition rather than in the acidic condition. (n = 3, mean ± SE). (C) Aminophenylboronate agarose (PBA) isolation of DAQ-modified α-syn in vitro. α-syn is co-precipitated with PBA only in the DA existing condition. (D) PBA isolation of DAQ-modified α-syn from the cell extracts of PC12-α-syn-Tet Off. In a Dox withdrawal condition (α-syn inducing), α-syn is co-precipitated with PBA (2nd lane). From a cell extract treated with aMT in order to inhibit endogenous catecholamine metabolism, little α-syn is co-precipitated with PBA (3rd lane). (E) Co-incubation with cysteine inhibits oxidation of DA and DOPA in vitro. DA or DOPA was incubated with or without cysteine in pH 7.8 buffer. (F) Cysteine suppresses DA or DOPA-related oligomerization of α-syn. (G) Continuous treatment of cysteine significantly decreases DA/α-syn-related cytotoxicity in PC12 cells (*p<0.01, ANOVA).

In the present study, we demonstrated that DA/DOPA are required to be oxidized to form α-syn oligomers. Indeed, recent studies revealed that the oxidization of DA and DOPA are responsible for oligomerization of α-syn. DA and DOPA are known to be oxidized to DAQ and DOPAQ in alkaline condition, and these are subsequently auto-oxidized to DAch and melanin. We investigated the association between DA/DOPA oxidation and α-syn oligomerization. The results showed an increased oxidation of DA and DOPA in pH dependent manner, especially in alkaline pH, and α-syn easily forms a soluble oligomer under alkaline conditions, suggesting that oxidized DA may enhance the oligomerization of α-syn. We also showed a direct interaction of oxidized DA with α-syn, and this interaction may be an initial

step of α-syn oligomerization under DA existing condition.

Cysteine, a scavenger for quinone and an antioxidant, decreased DA oxidation and α-syn oligomerization (Fig. 3 E and F). Furthermore, cysteine rescued the cells from DA/α-syn-related toxicity in PC12 cells (Fig. 3G). This result suggests that antioxidative compounds may be candidates as protective materials against DA/α-syn-related toxicity in PD pathogenesis.

Polyphenols are a structural class of natural and/or synthetic organic chemicals characterized by the presence of phenol structure. Most polyphenols have antioxidative activity,[38,39] and a number of polyphenols have been reported as anti-parkinsonian compounds.[40–43] CGA is a polyphenol that is abundant in coffee. We investigated the protective effect of CGA on DA/α-syn-
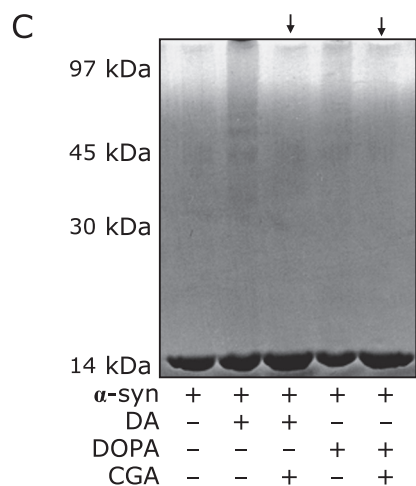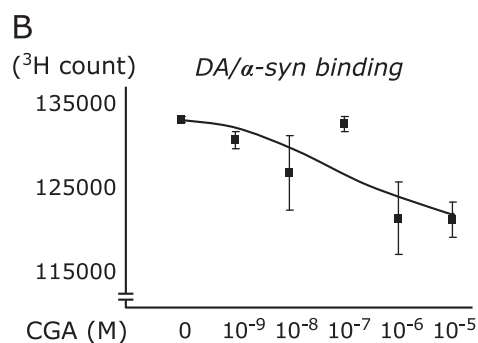
A

DA — + + — —   — + + — —
DOPA — — — + +   — — — + +
CGA — — + — +   — — + — +

10 h Incubation   20 h Incubation

B

(^{3}H count)

DA/α-syn binding

CGA (M) 0 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}

C

97 kDa
45 kDa
30 kDa
14 kDa

α-syn + + + + +
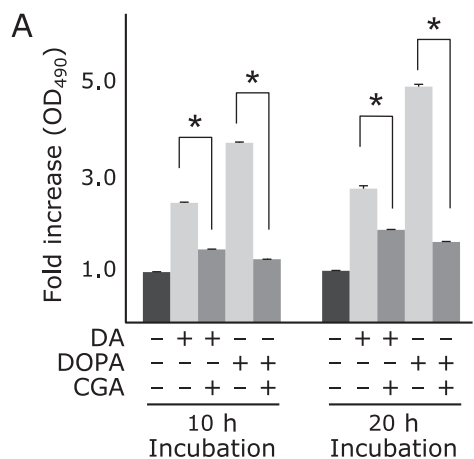DA — + + — —
DOPA — — — + +
CGA — — + — +

Fig. 4. CGA inhibits the interaction of DAQ with α-syn, the oxidation of DA and DOPA, and the oligomerization of α-syn. (A) Co-incubation of CGA dramatically inhibits oxidation of DA and DOPA *in vitro*. DA or DOPA was incubated with or without CGA in pH 7.8 buffer. (B) α-syn/ DA binding assay using ^{3}H-labeled DA. CGA inhibits the interaction between α-syn and oxidized DA at pH 7.8 in a dose dependent manner. (C) CGA suppresses DA or DOPA-related oligomerization of α-syn.

related toxicity. CGA showed not only inhibitory effects against DA oxidation (Fig. 4A) and direct interaction of oxidized DA to α-syn (Fig. 4B) but also α-syn oligomerization (Fig. 4C). As a result, CGA showed cytoprotective effects against α-syn toxicity in PC 12 cells (Fig. 5).

Taken together, the interaction of oxidized DA (or related compounds) with α-syn may be a novel therapeutic target for PD, and polyphenols including CGA are candidates as therapeutic
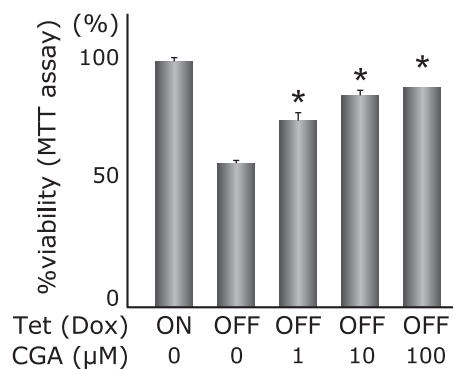

(%)

Tet (Dox) ON OFF OFF OFF OFF
CGA (μM) 0 0 1 10 100

Fig. 5. The protective effect of CGA on α-syn/DA-related toxicity in PC12 cells. PC12-α-syn-Tet Off cells were cultured under Dox-free condition (α-syn inducing) with several concentrations of CGA. The result shows the protective effect of CGA against α-syn/DA-related cytotoxicity in a concentration dependent manner (63.9% in 0 μM CGA, 75.6% in 1 μM CGA, 85.8% in 10 μM CGA, 89.1% in 100 μM CGA vs ON condition, respectively). Columns are the average of 6 independent experiments, and SE is indicated by the bar (*$p<0.01$, ANOVA).

or preventive materials for PD onset.

Abbreviations

DMEM   Dulbecco's modified eagle medium
EDTA   ethylenediaminetetraacetic acid
HCl   hydrogen chloride
PMSF   phenylmethylsulfonyl fluoride

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