

## Theaflavins stimulate autophagic degradation of $\alpha$ -synuclein in neuronal cells

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### ABSTRACT

*Given the recent epidemiological evidence showing an inverse correlation between black tea intake and the risk of Parkinson's disease, it is an intriguing possibility that theaflavins, major natural polyphenols contained in black tea, may have an anti-neurodegenerative action. In this communication, we show that theaflavins act stimulatingly on autophagy in neuronal cells. Treatment of murine Neuro2A cells with theaflavin-3,3'-digallate (TF-3,3') under the serum-free conditions resulted in stimulation of LC3-II expression and formation of LC3-positive, double-membrane autophagosomes. Under the same conditions, lysosomal activity was little affected by TF-3,3', while the upregulation of LC3-II by TF-3,3' was abrogated by 3-methyladenine treatment. Further studies revealed that TF-3,3' might stimulate degradation of Akt at proteasome, leading to suppression of mTOR phosphorylation and enhancement of autophagy. Finally, expression of the transfected  $\alpha$ -synuclein in these cells was significantly decreased by treatment with TF-3,3'. These results suggest that theaflavins' autophagy-stimulating action might account for the prophylactic effects of black tea on Parkinson's disease.*

### INTRODUCTION

Black tea is one of the most popular beverages consumed worldwide and has been thought to be beneficial for most chronic diseases, including coronary heart disease and different types of cancers [1]. In this context, numerous

studies have shown that theaflavins, major polyphenols contained in black tea, possess a variety of protective effects such as anti-oxidation, anti-cancer, anti-bacteria, and cardiovascular protection [2, 3]. Although it was previously reported that theaflavins were protective for the

rat cerebral ischemia model through suppression of inflammation [4], the effect of theaflavins on the nervous system is still obscure. By contrast, green tea has been shown to improve an age-related cognitive decline. Catechins, major polyphenols in green tea, were demonstrated to be neuroprotective in a variety of pathological models, such as those for Parkinson's disease (PD), Alzheimer's disease and ischemia/reperfusion injuries [5]. Thus, one may wonder theaflavins may also possess beneficial effects on neurodegenerative disorders. In this regard, it is intriguing to note that black tea intake is inversely correlated with the risk of PD [6].

In PD and related disorders, abnormal accumulation and fibril formation of  $\alpha$ -synuclein ( $\alpha$ -syn) may play a central role in the pathogenesis [7]. Supporting this notion,  $\alpha$ -syn fibrils were a major constituent of Lewy bodies in the PD brains [8] and overexpression of  $\alpha$ -syn in both a mouse model recapitulated Lewy body-like neuronal inclusions [9]. Furthermore, accumulated  $\alpha$ -syn may interfere with proteasome, mitochondria, and various signal transduction pathways [7]. Thus, it is reasonable to speculate that theaflavins might be protective against the cytotoxicity of  $\alpha$ -syn. In support of this notion, it was recently described that Black tea theaflavins inhibit formation of toxic amyloid- $\beta$  and  $\alpha$ -syn fibrils in vitro [10].

Besides direct effects of theaflavins on the aggregation of  $\alpha$ -syn, there might be other mechanisms underlying the protective effects of theaflavins on the neurotoxicity of  $\alpha$ -syn. Indeed, it is well characterized that  $\alpha$ -syn is degraded by autophagy [11, 12], and activation of macroautophagy by rapamycin (RAP) resulted in a decreased expression of  $\alpha$ -syn in the PD cellular model system [11]. It was also shown that  $\alpha$ -syn was specifically subjected to chaperone-mediated autophagy [12]. Moreover, mice lacking genes for regulatory molecules of autophagy, including ATG5 and ATG7, exhibited neurodegeneration, suggesting that the basic level of autophagy is indispensable to neuronal homeostasis [13, 14]. In this context, the main objective of the present study was to determine if theaflavins stimulate degradation of  $\alpha$ -syn through upregulation of the autophagy-lysosome pathway in murine neuronal cells.

## METHODS

### Reagents

Theaflavin-3,3'-digallate (TF-3,3') was purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-methyladenine (3-MA), ammonium chloride ( $\text{NH}_4\text{Cl}$ ) and RAP were obtained from Sigma (St. Louis, MO). The antibodies used in the study were: anti-LC3 from ABR (Golden, CO), anti-LC3B from NOVUS (Littleton, CO), anti-ATG5, anti-Beclin-1 and anti- $\beta$ -actin (AC-15) from Sigma, anti- $\alpha$ -syn (syn-1), anti-Akt and anti-ERK from BD Biosciences (Franklin Lakes, NJ), anti- $\alpha$ -syn LB509 from Wako Pure Chemical Industries, and anti-phospho-Akt, anti-phospho-mammalian target of RAP (mTOR) and anti-mTOR from Cell signaling (Beverly, MA), anti-ubiquitin from Chemicon (Temecula, CA), anti-hemagglutinin (HA) (12CA5) from Roche (Basel, Switzerland) and Alexa Fluor 488 conjugated anti-goat and anti-rabbit antibodies and Alexa Fluor 555 conjugated anti-mouse antibody (Molecular Probes, Eugene, OR).

### Cell cultures, expression plasmids and transfection

Neuro2A cells were cultured in Dulbecco's modified Eagle's medium (high glucose) containing 10% fetal calf serum (BioWest, Nuaille, France) and 1% v/v penicillin/streptomycin (Invitrogen, Carlsbad, CA) in a 5%  $\text{CO}_2$ /95% air atmosphere. These cells were previously used to investigate the roles of  $\alpha$ - and  $\beta$ -syn in neurodegeneration [15, 16]. Either pCEP4 (Invitrogen) with or without human wild type  $\alpha$ -syn cDNA [17] or pcDNA3 (Invitrogen) with or without mouse HA-Akt1 cDNA [18] (a kind gift from Dr. Kenneth Walsh at Boston University) was transfected to cells grown in either 6 or 12 multi-wells plates using LipofectAMINE 2000 (Invitrogen). The efficacy of transfection was approximately 70%.

### Immunoblot and co-immunoprecipitation analyses

Immunoblot analysis and immunoprecipitation experiments were done as previously described [17]. Briefly, cells were harvested and dissolved in lysis buffer: 1.0% Triton X-100, 50 mmol/L N-(2-hydroxyethyl) piperazine- $\text{N}^{\wedge}$ -(2-ethanesulfonic acid), 150 mmol/L NaCl, 10%

glycerol, 1.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L ethylene glycol bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 100 mmol/L sodium fluoride, and a protease inhibitor cocktail (Nacalai Tesque, Tokyo, Japan). The cells extracts were centrifuged for 10 minutes at 15,000 rpm and the supernatants were collected to measure the protein concentrations using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The insoluble fractions were resuspended in the lysis buffer and solubilized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The supernatants (detergent-soluble fractions) (10  $\mu$ g) and the corresponding volume of the detergent-insoluble fractions were subjected to analysis. For, immunoprecipitation, cell extracts (200  $\mu$ g) were preabsorbed with protein G-Sepharose (GE Healthcare) for 1 h, and the precleared lysates were incubated with either syn-1 or mouse IgG control (each 1  $\mu$ g) overnight at 4 °C followed by incubation with protein G-Sepharose. The immune complexes were then washed three times with the lysis buffer. The samples were then heated in the SDS sample buffer for 5 min and subjected to immunoblotting.

### Evaluation of cathepsin B activity

Measurement of cathepsin B activity was done as previously described [17]. Briefly, cells growing in sub-confluent conditions were harvested in buffer containing 50 mM HEPES (pH 7.4), 10 mM EDTA, and 10 mM NaCl, subjected to freezing and thawing to rupture cell membranous structures, and centrifuged at 15,000 rpm for 10 min. The supernatants (10  $\mu$ g) were then incubated either with benzyloxycarbonyl-Arg-Arg-Glu-amidomethyl-coumarin fluorogenic cathepsin B substrate (40  $\mu$ M, purchased from Chemicon). The enzymatic activities were assayed by continuous recording of the fluorescence activity released from fluorogenic substrate using Berthold Mithras LB940 microplate reader (Berthold, Bad Wildbad, Germany) for 1 h at 37 °C (excitation, 380 nm; emission, 460 nm), and the reaction rates were analyzed. The activities were described as arbitrary units/min/mg of protein.

### Immunofluorescence/laser scanning confocal microscopy (LSCM)

An immunofluorescence study was performed as previously described [19]. Briefly,

cells were inoculated on poly-L-lysine-coated glass coverslips, grown to 70% confluence, fixed in 4% paraformaldehyde for 30 minutes, and pretreated with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 minutes. Fixed cells were blocked with PBS containing 3% goat serum and 5% bovine serum albumin at room temperature. For staining, the cells were incubated overnight at 4°C with the primary antibody (or antibodies for double/triple staining). After washing, the cells were incubated with Alexa Fluor-conjugated secondary antibody (or antibodies for double/triple staining) (Invitrogen) for 1 hour at room temperature. In some experiments, cells were stained with 6-diamino-2-phenylindole dihydrochloride (DAPI) to stain the nucleus. Coverslips were mounted on the slides with Gel/Mount (Biomedica Corp., Foster City, CA) and imaged using a LSCM (FV1000; Olympus, Tokyo, Japan).

### Electron microscopy

Electron microscopic analysis was performed as previously described [19]. Briefly, cells were harvested using trypsin-EDTA and fixed by 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C for 2 h. After centrifugation, cells were washed with 0.1 M sodium cacodylate buffer three times. Cell pellets were obtained by centrifugation, post-fixed in 1% osmium tetroxide and 1% potassium ferrocyanide at room temperature for 2 h, and processed for embedding in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead nitrate and observed using a Hitachi H-7500 electron microscope.

### Statistical analysis

The values were compared by two-group t-tests and differences were considered significant for p-values were less than 0.05.

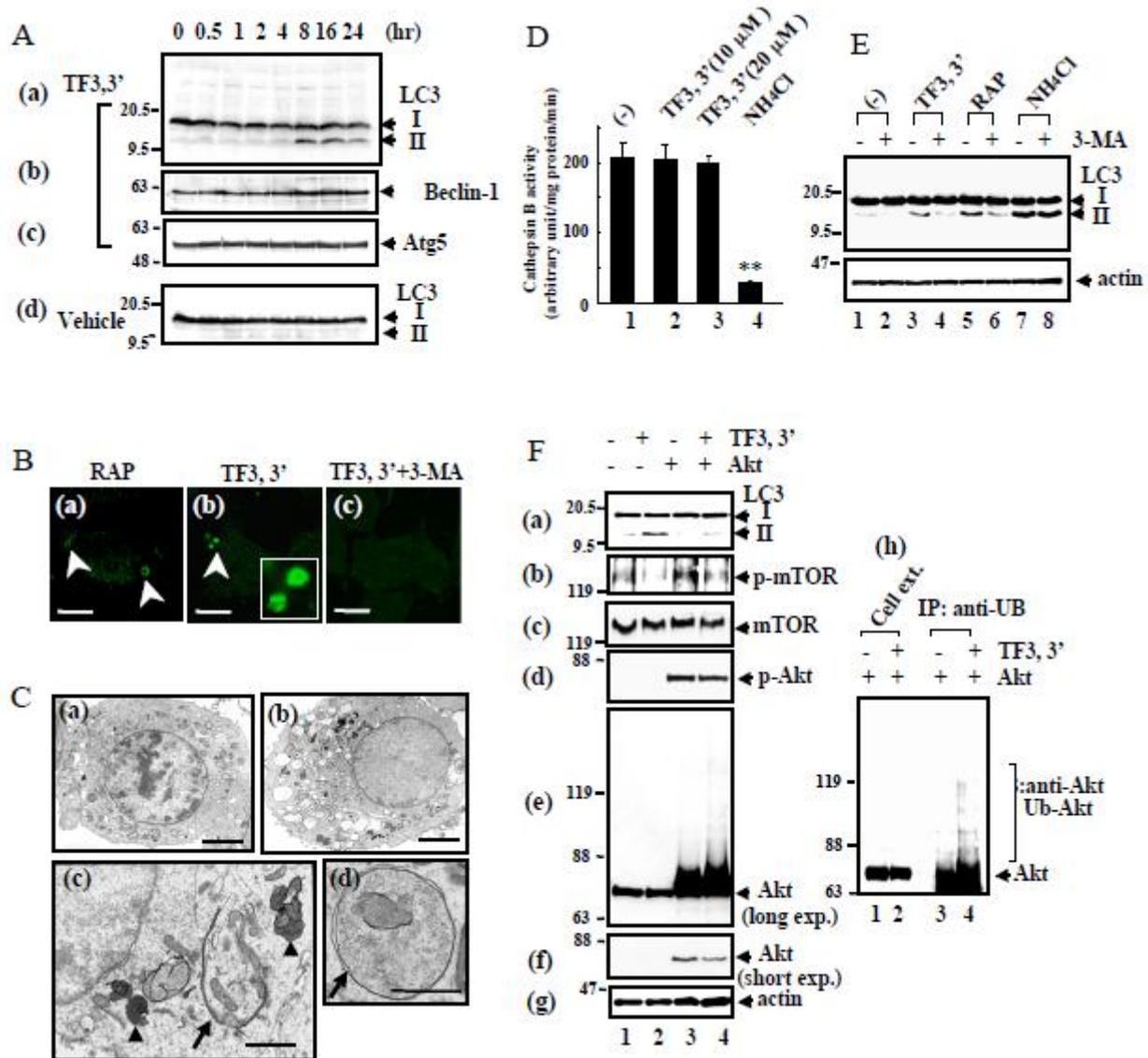
## RESULTS AND DISCUSSION

### TF-3,3' stimulates autophagosome formation in Neuro2A cells

Upon various inductive signals, autophagy starts with the appearance of the isolation membrane, followed by the formation of autophagosome and its fusion with lysosome where the contents are degraded [20]. During this process, cytoplasmic LC3 associates with

membrane of autophagosome. Since the cytoplasmic LC3 (LC3-I) and autophagosome-associated LC3 (LC3-II) exhibit differential mobility

on immunoblot analysis, LC3-II can be used as a marker protein for autophagosome formation [21].



**Fig. 1 TF-3,3' stimulates autophagy in Neuro2A cells**

- (A) Immunoblot analyses of LC3 (a and d), Beclin-1 (b) and Atg5 (c). Cells were treated with either TF-3,3' (a-c) or vehicle (0.2% ethanol) (d) under the serum-free conditions up to 24 hr. Cell extracts (10  $\mu$ g) were analyzed by immunoblotting. Similar results were obtained in three independent experiments.
- (B) Immunofluorescence/LSCM image of LC3 expression. Cells were treated with RAP (10  $\mu$ M) (a), TF-3,3' (10  $\mu$ M) (b) or TF-3,3' (10  $\mu$ M) plus 3-MA (10 mM) (c) for 12 hr, followed by staining with anti-LC3 antibody. Bars represent 10  $\mu$ m. Arrowheads indicate ring-like structures of autophagosomes. Inset shows a high magnification of the membrane ring-like structures in TF-3,3'-treated cells.
- (C) Representative electron micrographs of cells treated with TF-3,3' (10  $\mu$ M) (b, c, and d) or vehicle (0.1% ethanol) (a) for 12 hr. In TF-3,3'-treated cells, extensive formation of vacuoles and lysosomal structures are observed, whereas the nucleus is kept intact (b). In higher magnification (c), an isolated membrane which surrounds the mitochondria (arrow) and other cellular debris is observed in addition to electron-dense assembly of lysosome-like inclusions (arrowheads). Typical autophagosome with double membrane (arrow) is also found in TF-3,3'-treated cells (d). Fewer lysosomal structures are found in vehicle (0.2% ethanol)-treated cells (a). Bars represent either 4  $\mu$ m (a and b) or 1  $\mu$ m (c and d).
- (D) Measurement of cathepsin B activity. Cells were treated with vehicle (0.2% ethanol) (lane 1), TF-3,3' (10 and 20  $\mu$ M) (lanes 2 and 3) or NH<sub>4</sub>Cl (lane 4) for 8 hr, and cell extracts (10  $\mu$ g) were incubated at 37  $^{\circ}$ C with fluorogenic

cathepsin B substrate to measure the activity of cathepsin B. Data are shown as means  $\pm$  SD (n=4). \*\*:  $p < 0.01$  versus vehicle-treated cells

- (E) Immunoblot analysis of LC3. Cells were treated with vehicle (0.2% ethanol) (lanes 1 and 2), TF-3,3' (20  $\mu$ M) (lanes 3 and 4), RAP (lanes 5 and 6) or  $\text{NH}_4\text{Cl}$  (lanes 7 and 8) in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 3-MA (10 mM) for 8hr. Cell extracts (10  $\mu$ g) were analyzed by immunoblotting. Similar results were obtained in three independent experiments.
- (F) Immunoblot analyses of LC3, Akt, and mTOR. Cells were transfected with either pcDNA3 (lanes 1 and 2) or pcDNA3-HA-AKT (lanes 3 and 4) for 36 hr, followed by treatment with either vehicle (0.2% ethanol) (lanes 1 and 3) or TF-3,3' (20  $\mu$ M) (lanes 2 and 4) for 8 hr. Cell extracts (10  $\mu$ g) were harvested and analyzed by immunoblotting using anti-LC3 (a), anti-phosphorylated (p)-mTOR (b), anti-mTOR (c), anti-phosphorylated (p)-Akt (d), anti-Akt (e and f) or anti-actin (g). Note that panel f is a short exposure of panel e. In panel h, cells were transfected with pcDNA3-HA-AKT for 36 hr, followed by treatment with either vehicle (0.2% ethanol) (lanes 1 and 3) or TF-3,3' (20  $\mu$ M) (lanes 2 and 4) under the serum-free conditions for 8 hr. Cell extracts (200  $\mu$ g) were then immunoprecipitated with anti-ubiquitin antibody, followed by immunoblotting with anti-Akt. Cell extracts (10  $\mu$ g) were added as controls (lanes 1 and 2). Similar results were obtained in three independent experiments.

To determine if theaflavins stimulate LC3-II expression in Neuro2A cells, cells were treated with TF-3,3' (20  $\mu$ M) under the serum free conditions and expression of LC3 was monitored by immunoblot. The result showed that LC3-II was first detected at 8 hr post-treatment and the expression continued up to 24 hr post-treatment (Fig. 1A, a). Beclin-1 and Atg5 remained unchanged after TF-3,3' treatment (Fig. 1A, b and c). No LC3-II was induced when TF-3,3' was absent (Fig. 1A, d). LC3-II induction by TF-3,3' occurred in a dose dependent manner, but the toxicity of TF-3,3' was distinct at 40  $\mu$ M (data not shown). Therefore, 10~20  $\mu$ M concentrations of TF-3,3' were used under the serum free conditions in the majority of experiments. Similar concentrations of TF-3,3' were used in previous studies [21, 22]. Furthermore, TF-3,3' was the most potent to induce LC3-II compared to other theaflavin derivatives, including theaflavin, theaflavin-3-gallate and theaflavin-3'-gallate, (data not shown). Moreover, the comparative analysis using catechins and resveratrol with TF-3,3' indicated that TF3,3' was the most effective LC3-II inducer (data not shown).

Next, the effect of TF-3,3' on autophagosome formation was morphologically analyzed. In our preparations, treatment of cells with RAP for 8 hr resulted in the formation of LC3-II positive rings (Fig. 1B, a). Similar LC3-II positive rings were observed by TF-3,3' treatment (Fig. 1B, b), which was abrogated in the presence of 3-MA, an inhibitor of type III PI3 kinase which may play an important role during the initial phase of autophagy (Fig. 1B, c) [20]. By electron microscopy, lysosomal dense bodies and vacuoles were more obvious in TF-3,3'-treated cells than in

control cells (Fig. 1C, a and b). Despite of extensive autophagic changes in the cytoplasm, nucleus remained intact and no indication of apoptosis was seen in TF-3,3'-treated cells. At a high magnification, in addition to typical double-membrane autophagosome, a variety of autophagy-associated structures, including extended membranous cisternae (isolation membrane), assembly of dense lysosome-like inclusions and swollen mitochondria exhibiting abnormally layered membranes, were noted in TF-3,3'-treated cells (Fig. 1C, c and d). These findings suggested that the entire process of autophagy from the isolation membrane to autolysosomes was induced by TF-3,3' treatment in these cells.

### TF-3,3' may stimulate autophagy through Akt-mTOR pathway

Increased level of autophagosome formation is accounted not only by autophagy stimulation but also by autophagosome accumulation [20]. The latter is brought about by failure of fusion between autophagosome and lysosome due to lysosomal dysfunction [20]. To determine if lysosomal function is affected by TF-3,3' treatment, the activity of cathepsin B, a typical lysosomal cysteine protease, was evaluated (Fig. 1D). The result showed that the activity of cathepsin B remained unchanged after treatment with TF-3,3', whereas it was significantly suppressed by a lysosomal inhibitor  $\text{NH}_4\text{Cl}$  under the same conditions, suggesting that lysosomal function was little affected by the TF-3,3' treatment.

Then, to determine if the initial phase of autophagic process is essential to the induction of LC3-II expression by TF-3,3', the effect of 3-MA

was investigated. The result showed that induction of LC3-II by TF-3,3' was suppressed by co-incubation with 3-MA (Fig. 1E), being consistent with the result of morphological analysis (Fig. 1B). Similarly, 3-MA suppressed rapamycin-induced LC3-II expression. By contrast, NH<sub>4</sub>Cl-induced LC3-II expression was not affected by 3-MA. Essentially similar results were obtained when expression of Beclin-1 was knocked down by the siRNA strategy (data not shown). Collectively, it was suggested that autophagosome formation of TF-3,3' was induced through autophagy stimulation but not through accumulation of autophagosomes due to lysosomal dysfunction.

Mechanistically, it has been shown that mTOR-pathway may play a central role in a conventional autophagy. mTOR is an atypical serine/threonine kinase which functions as a sensor for cellular energy and amino acid levels, and negatively regulates the process of autophagy. Thus, rapamycin, an inhibitor of mTOR, can directly initiate the autophagy pathway as it occurs similarly in starved-cells. We speculated that TF-3,3' might suppress the Akt-mTOR pathway, leading to stimulation of autophagy because previous studies have shown that a variety of signaling molecules, including Akt, are negatively regulated by theaflavins [24], and because Akt is clearly situated upstream and stimulates phosphorylation of mTOR in various signal pathways, such as translational control [25] and insulin signaling [26].

To test this hypothesis, cells were transfected with either Akt expression vector or control vector prior to incubation with TF-3,3' and the effect of TF-3,3' on the activity (phosphorylation) of mTOR was evaluated by immunoblotting (Fig. 1F, a-g). As we expected, the ratio of phosphorylated- versus total-mTOR was significantly decreased in control vector-transfected cells by treatment with TF-3,3' (Fig. 1F, b and c, lanes 1 and 2). In Akt-transfected cells, it was shown that the activity (phosphorylation) of mTOR was concomitantly increased with that of Akt (Fig. 1F, b and d, lane 3), which was suppressed by TF-3,3' treatment and accompanied only by a weak induction of LC3-II expression (Fig. 1F, a, lane 4). These results suggest that downregulation of Akt-mTOR pathway may be involved in stimulation of autophagy by TF-3,3' treatment.

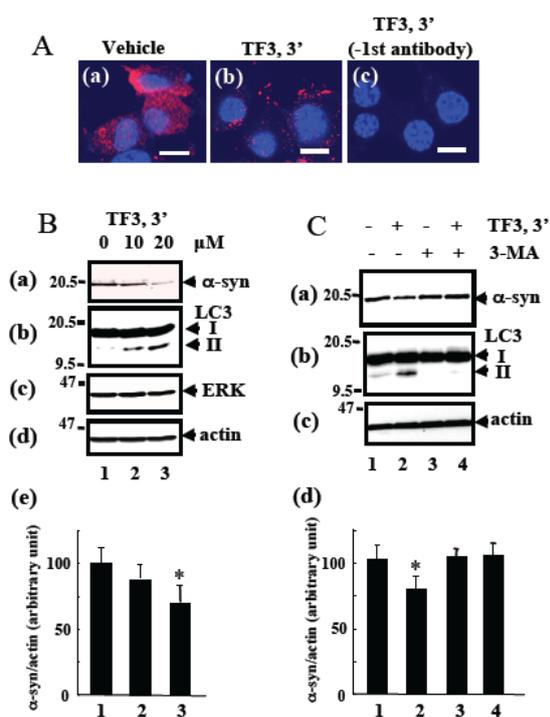
Although previous studies have shown that phosphorylation of Akt is suppressed by theaflavins, the underlying molecular mechanism has not been described [24]. In this regard, we consistently observed that treatment of the Akt transfected cells with TF-3,3' resulted in decreases of phosphoAkt and monomer form of total Akt, associated with higher molecular weight immunoreactivities (Fig. 1F, d-f, lanes 3 and 4), suggesting a possibility that ubiquitination of Akt was increased by treatment with TF-3,3'. To further confirm this possibility, the Akt-transfected cells with or without TF-3,3' treatment were harvested and cell extracts were immunoprecipitated with the anti-ubiquitin antibody, followed by immunoblotting with the anti-Akt antibody (Fig. 1F, h). The result showed that ubiquitinated Akt of a high molecular weight were detected more strongly in TF-3,3'-treated cells compared to vehicle-treated cells. Similar results were obtained when cell extracts were immunoprecipitated with the anti-HA antinody, followed by immunoblotting with the anti-ubiquitin antibody (data not shown). Thus, it was suggested that TF-3,3' stimulated degradation of Akt at the ubiquitin-proteasome system, leading to mTOR suppression. Such a mechanism is consistent with the observation that upregulation of LC3-II by TF-3,3' becomes apparent after several hours of treatment (Fig. 1A). Furthermore, it is interesting to note that TF-3,3' stimulated degradation of EGF receptor at the ubiquitin-proteasome system [23]. Together with our observation, one may speculate that degradation of some signaling molecules at the ubiquitin-proteasome system might be potentially stimulated by theaflavins.

### **TF-3,3' stimulates autophagic degradation of $\alpha$ -syn**

To determine if the intracellular protein levels of  $\alpha$ -syn could be decreased by elevated autophagy, the expression of  $\alpha$ -syn was monitored in cells after treatment with TF-3,3'. Since endogenous expression of  $\alpha$ -syn is negligible, human  $\alpha$ -syn was transfected to Neuro2A cells for 36 hr, followed by treatment with TF-3,3' under the serum-free conditions. Immunofluorescence microscopy showed that the staining for human  $\alpha$ -syn was less intense in TF-3,3'-treated cells than in the vehicle-treated

control (Fig. 2A). Consistent with this, immunoblot analysis showed that  $\alpha$ -syn was decreased in a dose dependent manner after TF-3,3' treatment, which was inversely proportional to LC3-II (Fig. 2B). Since mRNA expression of transfected  $\alpha$ -syn was little changed as demonstrated by reverse transcriptase-polymerase chain reaction (data not shown), it was unlikely that TF-3,3'-induced decrease of  $\alpha$ -syn was due to the down-regulation of its mRNA. In the similar context, ERK expression levels were comparable among treated and untreated cells (Fig. 2B, c). Further analysis showed that decrease of  $\alpha$ -syn protein expression by TF-3,3' was significantly suppressed by simultaneous treatment with 3-MA (Fig. 2C). These data support our hypothesis that TF-3,3' induced autophagy and reduced the intracellular protein level of  $\alpha$ -syn.

Fig.2



**Fig. 2 TF-3,3' stimulates autophagic degradation of  $\alpha$ -syn**

(A) Representative immunofluorescence/LSCM images of  $\alpha$ -syn expression: After transfection of  $\alpha$ -syn for 36 hr, cells were treated with either vehicle (0.1% ethanol) (a and c) or TF-3,3' (10  $\mu$ M) (b) for 12 hr. Cells were then fixed and stained for  $\alpha$ -syn using anti- $\alpha$ -syn LB509 (a and b). In panel c, primary antibody was omitted. Nuclei were simultaneously stained with 6-diamino-2-phenylindole dihydrochloride. The bar represents 10  $\mu$ m and all

figures are at the same magnification.

(B) Immunoblot analyses of  $\alpha$ -syn, LC3, ERK and actin. After transfection of  $\alpha$ -syn for 36 hr, cells were treated with either vehicle (0.2% ethanol) (lane 1) or TF-3,3' (10 and 20  $\mu$ M) (lanes 2 and 3) for 8 hr. Cell extracts (10  $\mu$ g) were harvested and immunoblot analyses were performed using syn-1 (a), anti-LC3 (b), anti-ERK (c) or anti-actin (d). Similar results were obtained in three independent experiments. In (e), the intensity of the band of  $\alpha$ -syn was quantified using BioMax 1D image analysis and corrected with that of actin. Data are shown as means  $\pm$  SD (n=3). \*: p<0.05 versus vehicle-treated cells.

(C) Immunoblot analyses of  $\alpha$ -syn, LC3 and actin. Cells were treated with either vehicle (0.2% ethanol) (lanes 1 and 3) or TF-3,3' (20  $\mu$ M) (lanes 2 and 4) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 3-MA (10 mM) for 8 hr. Cell extracts (10  $\mu$ g) were harvested and immunoblot analysis was performed using syn-1 (a), anti-LC3 (b), and anti-actin (c). Similar results were obtained in three independent experiments. In (d), the intensity of the band of  $\alpha$ -syn was quantified using BioMax 1D image analysis and corrected with that of actin. Data are shown as means  $\pm$  SD (n=3). \*: p<0.05 versus vehicle-treated cells.

### Theaflavins as prophylactic autophagy enhancers

Because up-regulation of autophagy might be a useful therapeutic strategy to remove intracellular aggregate-prone proteins, much attention has been paid to RAP and its analogue for their therapeutic potentials for various protein accumulation diseases. In neurodegenerative diseases, it was shown that RAP and its-derivative CCI-779 facilitated the excretion of Huntingtin in a drosophila and mouse model systems [27]. Moreover, novel compounds which have autophagy stimulating activities have been extensively exploited [28]. However, there are concerns that these reagents may be associated with unknown side effects considering that RAP has a potent immunosuppression effect [29]. The present study suggests that the autophagy stimulating property of theaflavins might be one possible mechanism accounting for the prophylactic effects of black tea on PD. Since dietary theaflavins are of benefit to health and there are no general safety concerns of side effects with ingestion of tea products, and since antioxidative activity of theaflavins should be also beneficial to the neurodegenerative

conditions, theaflavins might be promising as a novel autophagy enhancer to reduce the incidence of neurodegenerative disease. Currently, it is unclear how efficiently theaflavins may cross the blood-brain barrier and how quickly they are metabolized in the body. Nonetheless, it is possible that the present findings may serve as a basis for developing more suitable compounds for therapeutic purposes. Furthermore, future study may determine if our results obtained in a cell-based study are applicable to the in vivo model such as  $\alpha$ -syn transgenic mice, whereby the mode and dosage to maximize the beneficial effects of theaflavins on neuropathology will be critically investigated.

## ABBREVIATIONS

PD – Parkinson disease

syn – synuclein

RAP – rapamycin

TF-3,3' – theaflavin-3,3'-digallate

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3-MA – 3-methyladenine

NH<sub>4</sub>Cl – ammonium chloride

mTOR – Mammalian target of rapamycin

HA – hemagglutinin

LSCM – laser-scanning confocal microscope

## CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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