The role of quercetin on the survival of neuron-like PC12 cells and the expression of α-synuclein

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Abstract  
Both genetic and environmental factors are important in the pathogenesis of Parkinson's disease. As α-synuclein is a major constituent of Lewy bodies, a pathologic hallmark of Parkinson's disease, genetic aspects of α-synuclein is widely studied. However, the influence of dietary factors such as quercetin on α-synuclein was rarely studied. Herein we aimed to study the neuroprotective role of quercetin against various toxins affecting apoptosis, autophagy and aggresome, and the role of quercetin on α-synuclein expression. PC12 cells were pre-treated with quercetin (100, 500, 1,000 µM) and then together with various drugs such as 1-methyl-4-phenylpyridinium (MPP+); a free radical generator), 6-hydroxydopamine (6-OHDA; a free radical generator), ammonium chloride (an autophagy inhibitor), and nocardazole (an aggresome inhibitor). Cell viability was determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide (MTT) assay. Apoptosis was detected by annexin V-fluorescein isothiocyanate and propidium iodide through the use of fluorescence activated cell sorter. α-Synuclein expression was detected by western blot assay and immunohistochemistry. The role of α-synuclein was further studied by knocking out α-synuclein using RNA interference. Cell viability increased at lower concentrations (100 and 500 µM) of quercetin but decreased at higher concentration (1,000 µM). Quercetin exerted neuroprotective effect against MPP+, ammonium chloride and nocardazole at 100 µM. MPP+ induced apoptosis was decreased by 100 µM quercetin. Quercetin treatment increased α-synuclein expression. However, knocking out α-synuclein exerted no significant effect on cell survival. In conclusion, quercetin is neuroprotective against toxic agents via affecting various mechanisms such as apoptosis, autophagy and aggresome. Because α-synuclein expression is increased by quercetin, the role of quercetin as an environmental factor in Parkinson's disease pathogenesis needs further investigation.

Key Words: quercetin; Parkinson’s disease; α-synuclein; Levy body; PC12 cells; cell viability; cell death; neuroprotection

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Introduction
Both genetic and environmental factors are important in the pathogenesis of Parkinson's disease (PD) (Mullin and Schapira, 2015). A mutation on α-synuclein (αSyn) gene was first reported in familial cases of PD (Polymeropoulos et al., 1997). Moreover, abnormal inclusion of αSyn was found in the brains of both sporadic and hereditary cases of PD as an essential constituent of Lewy body (LB), a pathological hallmark of PD (Polymeropoulos et al., 1997; Spillantini et al., 1997). Thus αSyn is considered as one of the key molecules in PD and its clinical implication has been extensively studied (Cookson and Bandmann, 2010). αSyn mRNA expression was reported to be related to age, but few studies have focused on the influence of dietary factors on the expression of αSyn (Kim et al., 2004).

Inverse correlation between coffee or caffeine consumption and the risk of PD was reported in Western countries (Costa, 2010). In Asians, intake of tea was also related to lower risk of PD (Tan et al., 2003; Tanaka, 2011). (-)-Epigallocatechin-3-gallate (EGCG) is a tea flavonoid and was widely studied in the context of PD pathogenesis, demonstrating the beneficial role in experimental studies (Mandel et al., 2004). EGCG could affect remodeling of amyloid fibrils and mitigate αSyn pathology in the substantia nigra (Mandel et al., 2004; Bieschke et al., 2010).

Quercetin (Qc) is one of the most abundant flavonoids in common diet and more prevalent than EGCG but less commonly studied in association with PD pathogenesis (Mullie et al., 2007). Qc is a strong antioxidant and was widely studied to prevent oxidative stress in experimental...
models (Terao, 2009). Only a few studies were done in relation to PD pathogenesis, showing mild neuroprotective effect and dualistic role in which Qc was protective in early period of treatment and became harmful to the cells after prolonged exposure (Kääriäinen et al., 2008; Ossola et al., 2008). As oxidative stress is one of the leading mechanisms of cell death in PD, affecting αSyn toxicity, it would be interesting to investigate the relationship between Qc and αSyn expression.

Qc may affect other intracellular machineries such as proteasome degradation, heat shock protein expression, and apoptosis in addition to oxidative stress (Chen et al., 2005; Zanini et al., 2007). Other mechanisms such as autophagy and aggresome pathway drew the attention of the previous studies because inclusion formation of αSyn may be an essential procedure of LB (Spillantini et al., 1998; Sulzer, 2010; Wakabayashi et al., 2013). The role of Qc on these cellular mechanisms was not reported. Interestingly, a recent study showed that Qc and oxidized Qc inhibited αSyn fibrillization by the 1:1 covalent bindings of Qc with αSyn (Zhu et al., 2013).

In this study, we aimed to investigate the role of Qc on cell survival against various toxicants including the agents affecting the protein degradation pathway, which are thought to be involved in the pathogenesis of PD (Sulzer et al., 2010). We also planned to study αSyn expression by Qc and ubiquitination of αSyn because aggregated and ubiquitinated αSyn is a major component of LB (Kuzuhara et al., 1988).

Materials and Methods

Cell survival assay

Undifferentiated PC12 cells were purchased from ATCC (Manassas, VA, USA) and maintained in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). PC12 cells were plated (3 × 10^4) in a 96-well plate. Cell viability was determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) assay. After 24 hour treatment of Qc (Sigma) at different concentrations (100, 500, 1,000 µM), MTT solution (5 mg/mL in PBS) was added to the wells. MTT solution was removed after 3 hours of incubation and formazan precipitate was dissolved in 200 µL of dimethyl sulfoxide (DMSO; Sigma). Absorbance was measured at 540 nm (Beckman spectrophotometer; Beckman, Brea, CA, USA) using DMSO as a blank. Cell survival was expressed as the ratio of the optical density (percent survival).

To investigate mechanisms of Qc on cell survival, cells were treated for 24 hours with various toxicants such as 1-methyl-4-phenylpyridinium (MPP⁺; Sigma), 6-hydroxydopamine (6-OHDA; Sigma), ammonium chloride (NH₄Cl, AC; Sigma), and nocodazole (Nc; Sigma) in DMEM after Qc pre-treatment for 24 hours. MPP⁺ (a derivative of 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine, MPTP) and 6-OHDA damage neurons via various mechanisms such as free radical generation or mitochondrial dysfunction, which are frequently used in PD studies (Singer et al., 1990; Glinka et al., 1997). AC impairs autophagy via inter-vening lysosomal degradation whereas Nc interferes aggregate-lysosome fusion (Yamada et al., 2012). Fixed dose of various drugs such as MPP⁺ (0.75 mM), 6-OHDA (50 µM), AC (15 mM), Nc (100 mM) were used. z-VAD-fmk (zVf, 200 µM; Sigma) was used to rescue apoptosis after Qc treatment.

Flow cytometry

PC12 cells treated with Qc for 24 hours were harvested into tubes for flow cytometry. They were washed with phosphate buffer solution (PBS) and centrifuged for 2 minutes at 1,500 r/min. Annexin V-fluorescein isothiocyanate (AV; Sigma) was added to the cells on the ice for 30 minutes and then propidium iodide (Sigma; PI) was used. The cells stained with AV and PI were analyzed through the use of fluorescence activated cell sorter (FACS; BD Biosciences, San Hose, CA, USA). Those stained with only AV, only PI and both AV and PI were counted, respectively. The results were expressed as fractions.

Western blot analysis

Western blot for αSyn was done at 24 hours after Qc treatment. After PC12 cells treated with Qc were harvested and washed with ice-cold PBS and protein was extracted in a lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail (Sigma)]. Protein concentration was determined using bicinchoninate (BCA; Pierce, Rockford, IN, USA). Twenty micrograms of protein was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the nitrocellulose membrane. Incubation with primary antibodies [rabbit anti-αSyn antibody (1:1,000; Cell Signaling Technology, Beverly, MA, USA) and rabbit anti-β-actin (1:500; Sigma)] was performed overnight at 4°C. Incubation with an anti-rabbit-conjugated horseradish peroxidase antibody was performed at room temperature for 1 hour. Blots were visualized by chemiluminescence (Pierce). Band density was measured with a densitometer (GS700 model, BioRad, Hercules, CA, USA).

Immunofluorescent staining

For immunofluorescent staining, the cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature, treated with 4% normal goat serum in PBS with 0.2% Triton X-100 for 1 hour at room temperature, and then incubated with primary antibodies against αSyn (1:500) and ubiquitin (produced in mouse, 1:500; Chemicon, Temecula, CA, USA) at 4°C overnight. After washing the plates, cells were incubated with secondary antibodies, biotinylated anti-rabbit IgG and anti-mouse IgG (1:100; Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Visualization was done with Cy3-tagged streptavidin or FITC-tagged streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The images were photographed using confocal microscopy (Leica Microsystems, Wetzlar, Germany).
pairwise comparison (Turkey method) was done to compare two independent samples. The cutoff value of all the statistical analyses was set at $P = 0.05$.

Silencing aSyn gene
Silencing aSyn gene (Genebank Accession No. NM 019169) was done by small interfering RNAs (siRNAs; Allele Biotechnology, San Diego, CA, USA). Target sequence was CAG TGA GGC TTA TGA AAT and siRNAs was purchased from JCBiotech (Seoul, Korea). siRNA was cloned using SilenCircle™ RNAi system (Allele Biotechnology, San Diego, CA, USA). Briefly, top and bottom oligo (1 µg/µL, respectively) was prepared and annealed in annealing buffer. For ligation, annealed insert was incubated with pre-cut vector and T4 DNA ligase in cold block (4°C) for 12 hours. E. coli competent cells were mixed with the ligation product, which was treated with heat (42°C, 45 seconds). After heat treatment, the mixture was plated in ampicillin-containing media and incubated. Colonies containing transformed E. coli was selected and plasmids were harvested using mini-prep kit (Agilent Technology, Santa Clara, CA, USA). Plasmids were treated with restriction enzyme and self-ligated ones were eliminated after electrophoresis in 1.5% agarose gel. Transfection was done using AvantGene™2 transfection reagent (Allele Biotechnology) according to the manufacturer’s manual. Transfected cells were incubated for 72 hours and western blot assay was done to confirm the down-regulation of aSyn.

Statistical analysis
The results were analyzed by the SPSS 19.0 package for Windows (IBM Corp. Armonk, NY, USA). Data were presented as the mean ± SD. Analysis of variance (ANOVA) followed by post-hoc pairwise comparison (Turkey method) was done. Student’s t-test was done to compare two independent samples. The chi-square test was done to test distribution of samples. The cutoff value of all the statistical analyses was set at $P = 0.05$.

Results

Cell survival
Qc increased cell survival at the lower concentrations (Figure 1). The neurotoxicity of Qc became evident at 1,000 µM Qc, which was reverted by a pan-caspase inhibitor zVf (Figure 1). The pattern of cell survival after co-treatment of Qc and neurotoxic drugs including AC (an autophagy inhibitor) and Nc (an aggresome inhibitor) was similar, leaving that of survival after 6-OHDA treatment exceptional (Figure 2). In the pairwise comparison, the increase in cell survival by Qc was significant in the cells treated with MPP⁺ and AC treatment at 100 µM (Figure 2).

Cell apoptosis
FACS showed decreased fraction of apoptotic cells (stained with AV) at lower concentration of Qc (100 µM) and increased at highest concentration of Qc (1,000 µM; Figure 3A). The apoptotic fraction in the cells treated with MPP⁺ was larger than that in those treated with 6-OHDA in the absence of Qc. Apoptotic fraction was markedly decreased when Qc was added to the cells treated with MPP⁺ (Qc = 100 µM; Figure 3B). In the cells treated with 6-OHDA, co-treatment with Qc had no effect on apoptotic fractions (Figure 3C).

aSyn expression
The expression of aSyn increased by Qc but decreased even below the expression of control at 1,000 µM of Qc (Figure 4A1, A2). aSyn was rarely co-localized with ubiquitin (Figure 4B). Western blot assay confirmed a 90% down-regulation of aSyn using RNA silencing (Figure 5A). The survival pattern of aSyn-k0 was similar to that of the wild-type, with no significant difference in the cell survival at each concentration of Qc (Figure 5B, data not shown).

Discussion
Qc played a dual role on the survival of PC12 cells, while most previous studies only focused on either positive or negative effects of cell survival (Ansari et al., 2009; Galluzzo et al., 2009).

Because apoptotic fraction decreased in the presence of 100 µM of Qc, decreased apoptosis could be a mechanism of protective role of Qc, which was suggested in the previous studies (Figure 3) (Chen et al., 2005; Zanini et al., 2007). However, increased apoptosis at higher concentration of Qc could limit the usefulness of Qc to boost cell survival (Figure 3). The neurotoxic effect of 1,000 µM Qc was abolished by a pan-caspase inhibitor zVf (Figure 1). In the previous studies, caspase-3 activity increased after long-term exposure to higher concentration of Qc while it decreased after short-term exposure (Ossola et al., 2008). Thus, caspase-dependent cascade would be critical in the toxicity of Qc.

Qc was also effective for the cell death induced by autophagy and aggresome inhibitor, with maximal effect at 100 µM of Qc (Figure 2). The advantage of Qc on autophagy has been controversial in the previous studies, whereas no study...
Figure 2 PC12 cell survival after co-treatment of quercetin (Qc) and various drugs (MTT assay).
Qc treatment enhances cell survival against 1-methyl-4-phenylpyridinium (MPP⁺, 0.75 mM), ammonium chloride (AC, 15 mM) and nocodazole (Nc, 100 mM). 6-Hydroxydopamine (6-OHDA, 50 µM) induced cell death is not prevented by Qc. * Indicates analysis of variance, and ** indicates post hoc analysis by Turkey method. Data represent the mean ± SD of five independent experiments.

Figure 3 PC12 cell apoptosis after co-treatment of quercetin (Qc) and MPP⁺ or 6-OHDA (fluorescence activated cell sorter (FACS) analysis).
(A) A representative result of FACS. AV: Annexin V immunoreactive cells; PI: propidium iodide immunoreactive cells; AV + PI: annexin V and propidium iodide immunoreactive cells. (B) Early apoptotic fraction increases at higher concentration of quercetin (Qc) (C, D). In the baseline (Qc = 0 µM), apoptosis is more dominant with 1-methyl-4-phenylpyridinium 1-methyl-4-phenylpyridinium (MPP⁺) treatment than 6-hydroxydopamine (6-OHDA) (chi-square test, *P < 0.05). Co-treatment of Qc and MPP⁺ decreases apoptotic fraction at 100 µM of Qc (analysis of variance, #P < 0.05). In the cells treated with 6-OHDA, co-treatment of Qc has no effect. Each experiment was performed in triplicate.
Figure 4 α-Synuclein (aSyn) expression in PC12 cells by quercetin (Qc).
(A) Western blot shows a constitutional expression of aSyn of control cells (A1), in which the ratio of aSyn to actin increases up to 500 µM of Qc (A2). Each experiment was performed in triplicate. (B) Cells treated with 100 µM of Qc are stained with aSyn (a) and ubiquitin (b) antibody to show rare co-localization (c). Red = aSyn; green = ubiquitin. Scale bar: 50 µm.

Figure 5 Effect of RNA silencing of α-synuclein (aSyn) on PC12 cell survival following quercetin (Qc) treatment.
(A) Western blot confirms the knockout of aSyn (aSyn-ko). (B) Cell survival after Qc treatment in aSyn-ko cell line shows similar pattern to that in the wild type cells (see Figure 1; * indicates analysis of variance). Data represent the mean ± SD of five independent experiments.
was done on the role of Qc on aggresome (Psahoulia et al., 1999; Ossola et al., 2009). Our results suggested that Qc could modulate key molecular mechanisms associated with formation of aggregates (LB; Sulzer et al., 2010). In contrast to the previous study on the role of Qc on 6-OHDA treated cells, Qc failed to increase the survival of PC12 cells treated with 6-OHDA (Figure 2) (Ossola et al., 2008). Although previous studies suggested that 6-OHDA toxicity was more closely related to apoptosis than MPP+ toxicity, FACS analysis showed that cells treated with MPP+ were more apoptotic than those with 6-OHDA in the baseline without Qc (Figure 3) (Choi et al., 1999). The apoptotic fraction associated with MPP+ treatment was successfully rescued by 100 µM of Qc, which was not observed in the cells treated with 6-OHDA. Thus, the neuroprotection by 100 µM of Qc on the cells treated with MPP+ can be closely related to reduced apoptosis (Figures 2, 3). However, the role of Qc on deceasing apoptotic fraction was lost even in the cells treated with MPP+ at higher concentration of Qc (1,000 µM), which reminded the dualistic role of Qc (Ossola et al., 2008). Aggravated apoptosis at higher concentration of Qc may be critical (Figures 1, 3A).

As the formation of aggregation of aSyn is a core feature of PD, the relationship between aSyn and Qc was studied (Polymeropoulos et al., 1997; Spillantini et al., 1997). Western blot assay showed increased aSyn expression by Qc treatment (Figure 4). Qc was previously reported to prevent fibrillation of aSyn, but up-regulation of aSyn by Qc was not reported (Zhu et al., 2013). The normal function of aSyn is not fully understood except few constitutional roles such as helping the formation of SNARE or vesicle trafficking (Chandra et al., 2005; Cooper et al., 2006). Increased expression of aSyn with beneficial role was demonstrated in the cells treated by drugs or mutant cells with duplicated aSyn gene (SNCA) (Leng et al., 2006; Kim et al., 2008). However, SNCA duplication or triplication resulted in PD and increased aSyn expression was more considered as being detrimental to the cells (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibañez et al., 2004; Sala et al., 2013). In some studies, aSyn exerted neuroprotection at nanomolar concentration but became toxic or lost its beneficial role at micromolar concentration (Seo et al., 2002; Kim et al., 2013).

In this study, the expression of aSyn remarkably increased at lower concentration of Qc (50–500 µM) but was even below the level of constitutional expression at 1,000 µM of Qc, which was a dualistic role of Qc on aSyn expression (Figure 1). As the pattern of aSyn expression after Qc treatment was similar to that of cell survival, we performed aSyn knockout by RNA interference to investigate the contribution of aSyn in cell survival (Figure 5). Down-regulation of aSyn did not affect cell survival in our experiments. These results may suggest a non-critical role of aSyn on the survival of cells treated with Qc.

Pathologic aSyn associated with LB pathology is usually modified by posttranslational mechanisms such as phosphorylation, ubiquitination or nitration (Beyer and Ariza, 2013). In ubiquitination, ubiquitin is attached to aberrant or denatured proteins and ubiquitinated proteins are degraded by proteasome (Ristic et al., 2014). To explore ubiquitination of aSyn, we performed immunofluorescence staining. The results showed only rare co-localization of aSyn and ubiquitin (Figure 4). Thus, aSyn over-expressed by Qc treatment was not ubiquitinated, which was also suggestive of non-fatal nature of increased aSyn. Qc is ubiquitous in common diets and its bioavailability is better than previously assumed (Bischoff, 2008; Ossola, 2009). In our experiment, Qc was beneficial at acceptable concentration. Thus, although the therapeutic efficacy of Qc was questioned in some experimental models for neurodegenerative diseases, as it could touch various cellular machineries such as apoptosis, autophagy, aggresome, and aSyn expression, it is necessary to further study Qc as a significant dietary factor related to the pathogenesis of PD (Bischoff, 2008; Ossola, 2009).

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Conflicts of interest: None declared.

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CORRECTION

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