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Epigallocatechin-3-gallate (EGCG) inhibits aggregation of pulmonary fibrosis associated mutant surfactant protein A2 via a proteasomal degradation pathway

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ABSTRACT

Background/aims: Epigallocatechin-3-gallate (EGCG), a major catechin found in green tea, plays an important anti-tumor role and is involved in various other biological processes, such as, neuroprotection by prevention of aggregation of misfolded proteins generated because of genetic defects. Surfactant protein A2 mutations (G231V and F198S) have been identified to be associated with pulmonary fibrosis and lung cancer, and these mutations cause protein aggregation, instability as well as secretion deficiency. The present study focused on investigating the inhibitory effects of EGCG on aggregation of mutant SP-A2 and elucidating the potential mechanisms underlying this action.

Methods: Wild-type and mutant SP-A2 were transiently expressed in CHO-K1 cells. The aggregated and soluble proteins were separated into NP-40-insoluble and NP-40-soluble fractions. Protein stability was validated by chymotrypsin limited proteolysis assay. Western blot and RT-PCR were used to determine the protein and mRNA expression level, respectively.

Results: Mutant SP-A2 alone or wild-type SP-A2 co-expressed with G231V formed NP-40-insoluble aggregates in CHO-K1 cells. EGCG significantly suppressed this aggregation and alleviated mutant SP-A2 accumulation in the ER. When combined with 4-PBA, EGCG treatment completely blocked mutant SP-A2 aggregate formation. Though secretion of mutant protein was not affected, EGCG facilitated protein instability in both wild-type and mutant protein. Importantly, MG132, a proteasome inhibitor, reversed EGCG-induced aggregate reduction.

Conclusions: EGCG inhibits aggregation of misfolded SP-A2 via induction of protein instability and activation of proteasomal pathway for aggregate degradation.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive lung disease that primarily occurs in older adults, is characterized by shortness of breath and cough, and has no effective treatment currently (Blackwell et al., 2014; Fernandez and Eickelberg, 2012). Recent genetic studies have revealed that mutations in proteins, such as surfactant protein C (SP-C) and surfactant protein A2 (SP-A2), are associated with development of IPF (Nogee et al., 2001; Wang et al., 2009). Subsequent studies have found that these mutations resulted in protein misfolding and retention within the endoplasmic reticulum (ER) lumen of alveolar type II cells, thereby reducing protein secretion (Thurm et al., 2013; Song et al., 2012). These results suggested that chronic aggregation of misfolded proteins could be recognized as a potential marker of alveolar type II cells in IPF patients (Romero and Summer, 2017). Therefore, it has been highlighted that accumulation of misfolded proteins is a pathological feature of IFP similar to other age-related degenerative disease, such as Parkinson's and Alzheimer's disease (Chung et al., 2013). Therefore, targeting the misfolding of mutant protein or inhibition of aggregation of mutant protein might act as a potential therapy against IFP.

Epigallocatechin-3-gallate (EGCG) is a naturally-occurring small

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molecule, which is the most abundant and an active component of green tea (Kanwar et al., 2012). An increasing number of reports have expounded its anti-tumor, anti-inflammation, anti-aging, cardiovascular, neural protection, and several other properties (Song et al., 2018). For example, EGCG significantly suppressed cancer cell proliferation, invasion, and angiogenesis in breast cancer patients (Zhang et al., 2012; Lecumberri et al., 2013). EGCG also plays a crucial role in preventing the aggregation of Tau protein, which caused irremediable neuronal cell death and led to Alzheimer's disease (Gueroux et al., 2017). In addition, EGCG has recently been reported to contribute to the prevention of various airway disease, such as asthma, by several mechanisms, including binding to proinflamatory chemokine and inhibiting inflammatory cell recruitment, as well as inhibiting Epithelial-mesenchymal transition (EMT) (Yang et al., 2018; Liang et al., 2011).Importantly, recent studies have shown that EGCG inhibited radiation- or chemical-induced pulmonary fibrosis in adult rats (You et al., 2014; Sriram et al., 2009; Dona et al., 2003). As we previously showed, SP-A2 mutations (G231V and F198S), which are associated with pulmonary fibrosis, caused SP-A2 to form detergent-insoluble protein aggregates in cells (Song et al., 2012). However, whether EGCG could alleviate the aggregation of mutant SP-A2 and has a potential benefit in pulmonary fibrosis therapy that still needs to be determined.

In this study, we aimed to clarify the critical roles of EGCG in reducing SP-A2 mutant protein aggregation. We found that EGCG significantly reduced SP-A2 mutant protein aggregation and stability. Our results further revealed that EGCG might promote the proteasomal pathway to enhance the degradation of the aggregates of mutant SP-A2 protein.

2. Materials and methods

2.1. Reagents and antibodies

DMEM/F12, fetal bovine serum (FBS), and transfection reagent were obtained from GIBCO (Thermo Scientific, Rockford). Antibodies against Myc, calreticulin (H-170), and Bip were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-V5 mouse monoclonal antibody (R960-25) was purchased from Invitrogen. IRDye 680RD or IRDye 800CW-conjugated secondary antibody was obtained from LI-COR Biosciences (LI-COR, Lincoln). EGCG and other reagents were obtained from Sigma-Aldrich (St Louis, MO), unless otherwise indicated.

2.2. Cell culture and transfection

CHO-K1 cells (CCL-61) were purchased from the American Type

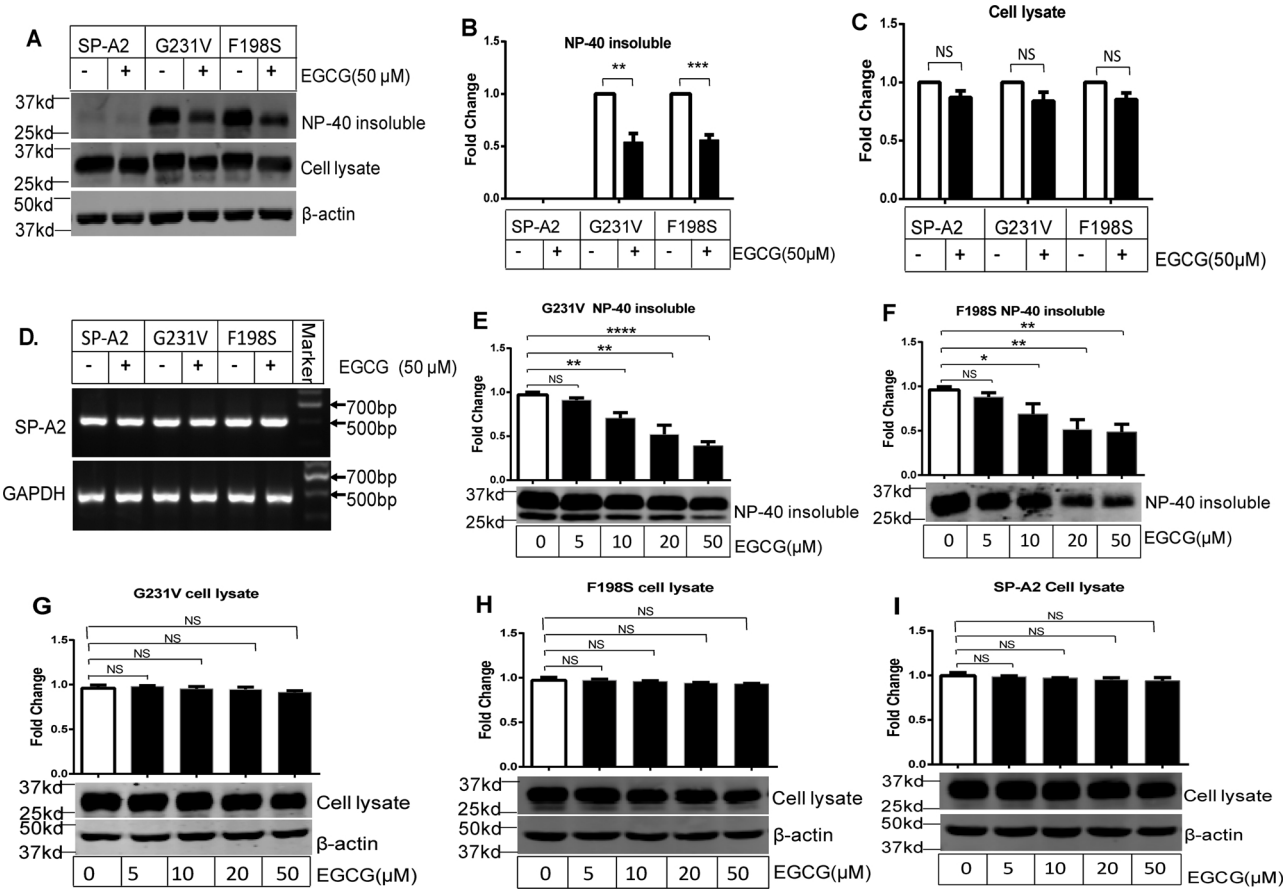

Fig. 1. EGCG decreased detergent-insoluble aggregates of mutated SP-A2 protein in CHO-K1 cells. (A) CHO-K1 cells were transiently transfected with V5-tagged SP-A wild-type, G231V, and F198S variants' full-length cDNA plasmids. Twenty four hours after transfection, EGCG were added to the cells for 48 h before the cells were harvested, divided into NP-40-soluble and -insoluble fractions, and equal amounts of total protein were subjected to SDS-PAGE followed by immunoblot analysis using a monoclonal antibody that recognizes the V-5 epitope. NP-40-insoluble fraction (B) and NP-40-soluble fraction (C) in cell lysate, **P < 0.01 and ***P < 0.001 by unpaired *t*-test. (D) CHO-K1 cells were transiently transfected with V5-tagged SP-A wild-type, G231V, and F198S variants' full-length cDNA plasmids. Twenty-four hours after transfection, EGCG, at the indicated concentrations, was added to the cells for 48 h before the cells were harvested, total RNA was extracted, and RT-PCR was performed using primers specific to SP-A2 and GAPDH gene. After CHO-K1 cells were treated with different concentrations (0, 5, 10, 20, 50 μM) of EGCG, NP-40 insoluble aggregates of G231V (E) or F198S (F) and cell lysates from G231V(G), F198S (H), and wild-type (I) SP-A2 are shown. Data are shown as the mean ± S.D. for at least three independent transfection experiments. * P < 0.05, ** P < 0.01, **** P < 0.0001 by one-way ANOVA; NS, no significance.

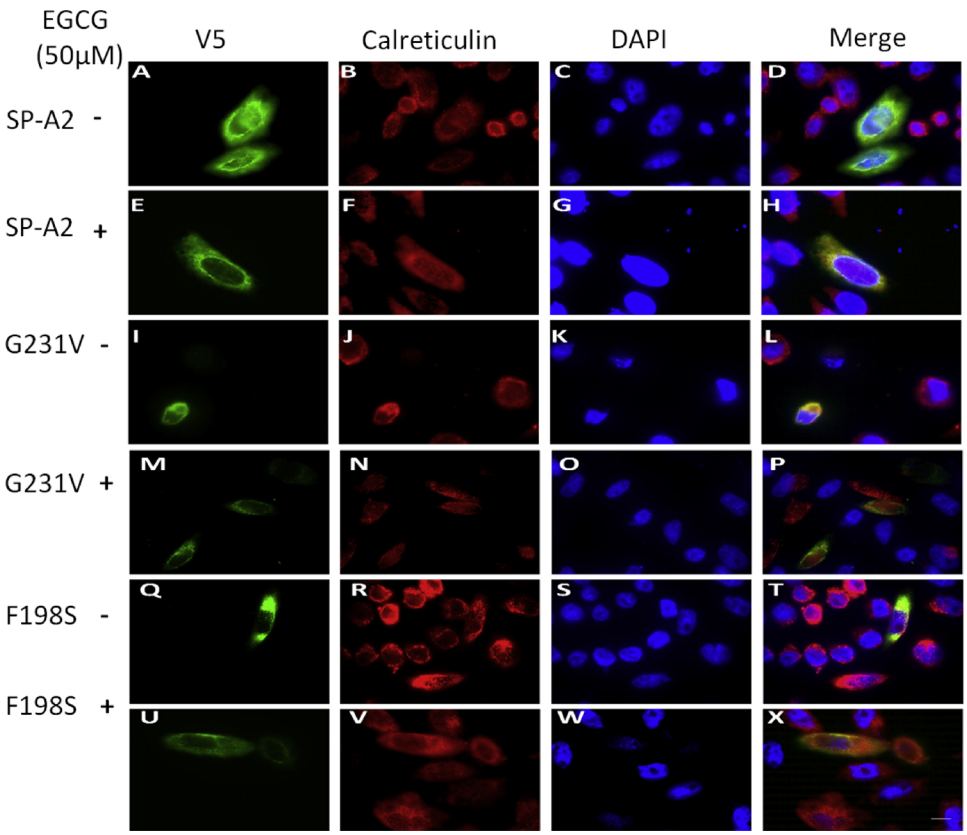

Fig. 2. EGCG prevented mutant SP-A2 accumulation in the ER. CHO-K1 cells were transiently transfected with SP-A2 wild-type, G231V, or F198S variants. After 24 h, cells were treated with 50 μM EGCG for additional 48 h, then fixed with 4% PFA. Cells were subjected to immunofluorescence staining using anti-V5 and anti-calreticulin antibodies to localize SP-A2 and the ER, respectively. EGCG reduced ER accumulation of SP-A2 (G231V) (M–P) and SP-A2 (F198S) (U–X). Scale bar, 10 μm.

Culture Collection and maintained in DMEM/F12 supplemented with 5% FBS, 100 units of penicillin, and 100 μg of streptomycin, and incubated at 37 ℃ in presence of 5% CO_2. Cells were transiently transfected with different constructs using FuGENE HD Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. Plasmid construction and mutagenesis were conducted as described previously (Song et al., 2012).

2.3. SDS-PAGE and immunoblot analysis

Protein concentrations of the cell lysates were determined by BCA protein assay (Thermo Scientific, Rockford, IL), according to the manufacturer's protocol. Protein extracts were electrophoresed on 10% SDS-PAGE and analyzed by immunoblotting as previously reported (Song et al., 2012). The specific bands were detected by SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL).

2.4. NP-40-soluble and NP-40-insoluble fractionation assay

After transfection for 48 h, CHO-K1 cells were harvested in lysis buffer (100 mM NaCl, 50 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 0.5% (v/v) NP-40 with protease inhibitor cocktail (Roche), collected in a 1.5 ml microcentrifuge tube and incubate in a shaker at 4 ˚C for 30 min. Cell lysates were sedimented at 16,000 g for 10 min at 4 ˚C. The supernatants were stored as the NP-40-soluble fraction. Pellets were washed with lysis buffer and solubilized using 2x SDS buffer (125 mM Tris-HCl, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.01% bromophenol blue) and stored as the NP-40-insoluble fraction (Yang et al., 2014).

2.5. Chymotrypsin limited proteolysis assay

CHO-K1 cells were harvested using lysis buffer without protease inhibitors (100 mM NaCl, 50 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 0.5% (v/v) NP-40). A mixture of 1:50 ratio (w/w) of α-chymotrypsin (dissolved in 50 mM Tris-HCl (pH 8), 100 mM NaCl) to protein was used for each single time point for the SP-A2 variants. Chymotrypsin was added at the different times and reactions were stopped simultaneously by the addition of SDS loading buffer and heating at 100 ˚C for 5 min. Digested peptides were visualized on 15% SDS-PAGE.

2.6. Immunofluorescence microscopy

Immunofluorescence staining was performed as described previously (Song et al., 2012). Briefly, CHO-K1 cells were fixed in 4% paraformaldehyde (PFA) followed by permeabilization and blocking. Primary antibodies against V5 and calreticulin were used to detect V5-tagged SP-A2 andER, respectively. Alexa-488 or 594-conjugated secondary antibody (Molecular Probes) were used and images were obtained by an Olympus FV1000 confocal microscope. Representative results are shown for at least three transfection experiments.

2.7. Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software, Inc., La Jolla, CA). Data are expressed as the mean ± S.D. The statistical significance of the differences between the means of groups was determined by one-way ANOVA or unpaired two-tailed t-tests. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. EGCG decreased the levels of detergent-insoluble aggregates of SP-A2 G231V and F198S variants

Two mutations of SP-A2, G231V and F198S, have previously been identified to be associated with pulmonary fibrosis and lung cancer

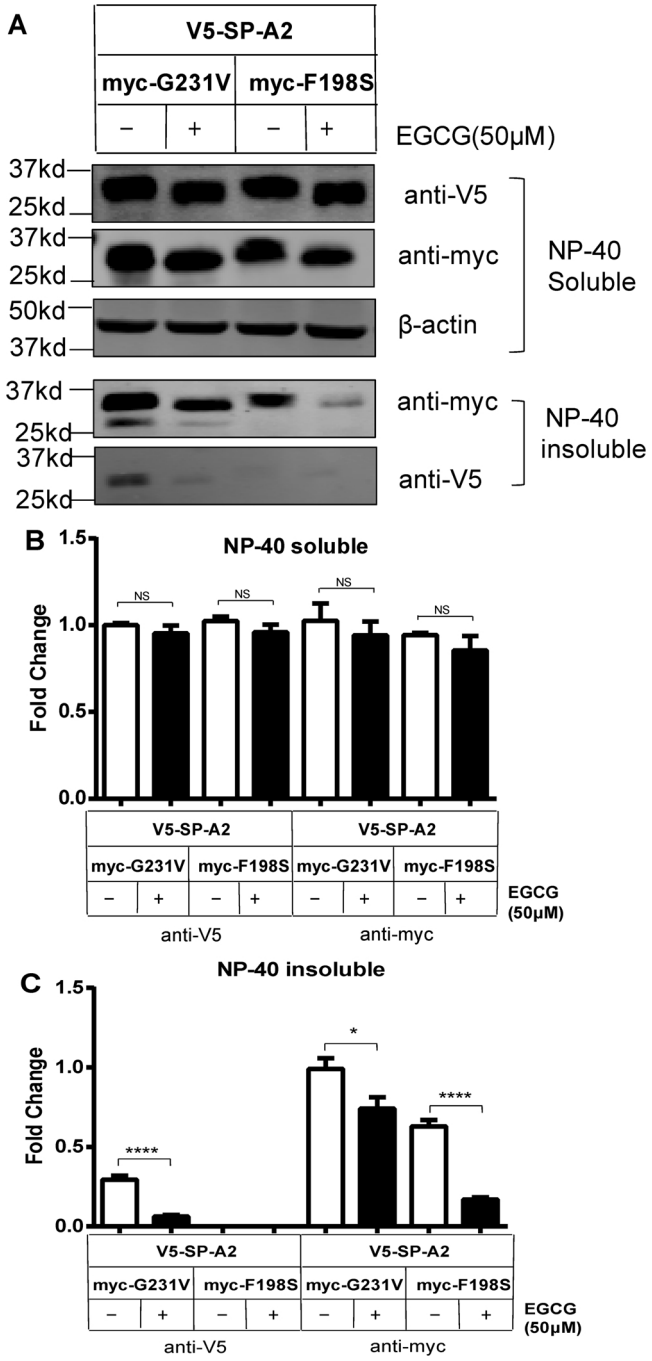

Fig. 3. EGCG reduced wild-type SP-A2 aggregates caused by co-expression with G231V variant. CHO-K1 cells were transiently co-transfected with V5-tagged SP-A2 and SP-A2 G231V or F198S variant tagged with myc. Twenty four hours later the medium was changed, treated the cells were treated with EGCG for 48 h, and then, equal amounts of total protein from cell lysate and NP-40-insoluble (A) were subjected to SDS-PAGE followed by immunoblot analysis using a monoclonal antibody that recognizes either the V-5 epitope or the myc-epitope. Data were quantified in NP-40-soluble (B) and -insoluble fractions (C) and shown as the mean ± S.D. for at least three independent transfection experiments. * $P < 0.05$, **** $P < 0.0001$ by unpaired *t*-test; NS, no significance.

(Wang et al., 2009). These mutations lead to deficiency in protein sialylation and secretion as well as formation of detergent-insoluble aggregates in cells (Song et al., 2012). To determine the effects of EGCG on the mutations, plasmids containing SP-A2 wild-type, G231V or F198S variants were transfected into CHO-K1 cells, which were then treated with EGCG at different concentrations ranging from 0 to 50 μM (0, 5, 10, 20, and 50 μM). The results showed that both mutant SP-A2 variants formed the NP-40 insoluble aggregates in CHO-K1 cells, and EGCG (50 μM) treatment decreased the levels of these aggregates (Fig. 1A and B) in a dose-dependent manner (Fig. 1E and F). However, EGCG did not significantly affect the NP-40 soluble fraction of both wild-type and mutant variants of SP-A2 (Fig. 1A and C, G–I). We further examined the SP-A2 mRNA levels by RT-PCR (sequence of primers shown in Table. S1) and found that EGCG treatment did not change SP-A2 mRNA transcription level (Fig. 1D), suggesting that EGCG-induced reduction of mutant SP-A2 aggregates might be not caused by alteration in mRNA levels.

3.2. EGCG alleviated mutant SP-A2 accumulation in the ER

We previously showed that SP-A2 G231V and F198S mutant proteins distributed in the peri-nuclear region, with most retention in the ER (Song et al., 2012). Since treatment with EGCG reduced mutant SP-A2 protein secretion, we wondered whether EGCG could prevent mutant SP-A2 accumulation in the ER. We transiently expressed V5-tagged wild-type and mutant SP-A2 variants in CHO-K1 cells and then used anti-V5 monoclonal antibody and anti-calreticulin polyclonal antibody to identify whether mutant SP-A2 proteins were still retained in the ER after EGCG treatment. Immunofluorescence staining showed that accumulation of both G231V and F198S mutant proteins in the ER in presence of EGCG was lower (Fig. 2M–P, U–X) compared to that without EGCG treatment (Fig. 2I–L, Q–T). In contrast, accumulation of wild-type SP-A2 protein did not show significant difference before and after EGCG treatment (Fig. 2A–H).

3.3. EGCG reduced wild-type SP-A2 aggregates formed after co-expression with G231V mutation

Previous studies have reported that SP-A2 G231V variant, but not F198S variant, could affect the solubility of wild-type SP-A2 variant when they were co-expressed in cells (Song et al., 2012). We confirmed that wild-type SP-A2 formed NP-40-insoluble aggregates when the CHO-K1 cells were co-transfected with V5- tagged SP-A2 and myc-tagged SP-A2 G231V. However, SP-A2 F198S did not show such dominant negative effect when co-expressed with wild-type SP-A2. Interestingly, EGCG treatment significantly reduced the aggregates of wild-type SP-A2 formed after co-expression with G231V variant (Fig. 3A (bottom panel lane 1–2) and 3C). Meanwhile, EGCG did not affect the NP-40-soluble fraction of wild-type SP-A2 variant (Fig. 3A and B).

3.4. Combined treatment of EGCG and 4-PBA completely blocked mutant SP-A2 aggregate formation

Our previous studies suggested that 4-PBA, a chemical chaperone, partially alleviated aggregates of the mutant SP-A2. Intriguingly, combined treatment of EGCG and 4-PBA synergistically abolished NP-40-insoluble aggregates of SP-A2 G231V (Fig. 4A and C) and F198S (Fig. 4B and D) variants in CHO-K1 cells. However, EGCG, in combination with 4PBA, did not alter the NP-40-soluble fractions of mutant SP-A2 variants, G231V (Fig. 4A) and F198S (Fig. 4B).

3.5. EGCG did not promote mutant protein secretion

We had reported previously that SP-A2 mutations led to protein sialylation and secretion deficiency caused by impairment of protein dimer/trimer assembly, and 4-PBA treatment could restore this deficiency to a certain extent. However, EGCG treatment did not alter mutant SP-A2 G231V and F198S secretion. Surprisingly, we observed reduction in secretion of wild-type SP-A2 in presence of EGCG (Fig. 5A), which was dose-dependent (Fig. 5B). Accordingly, we found that EGCG

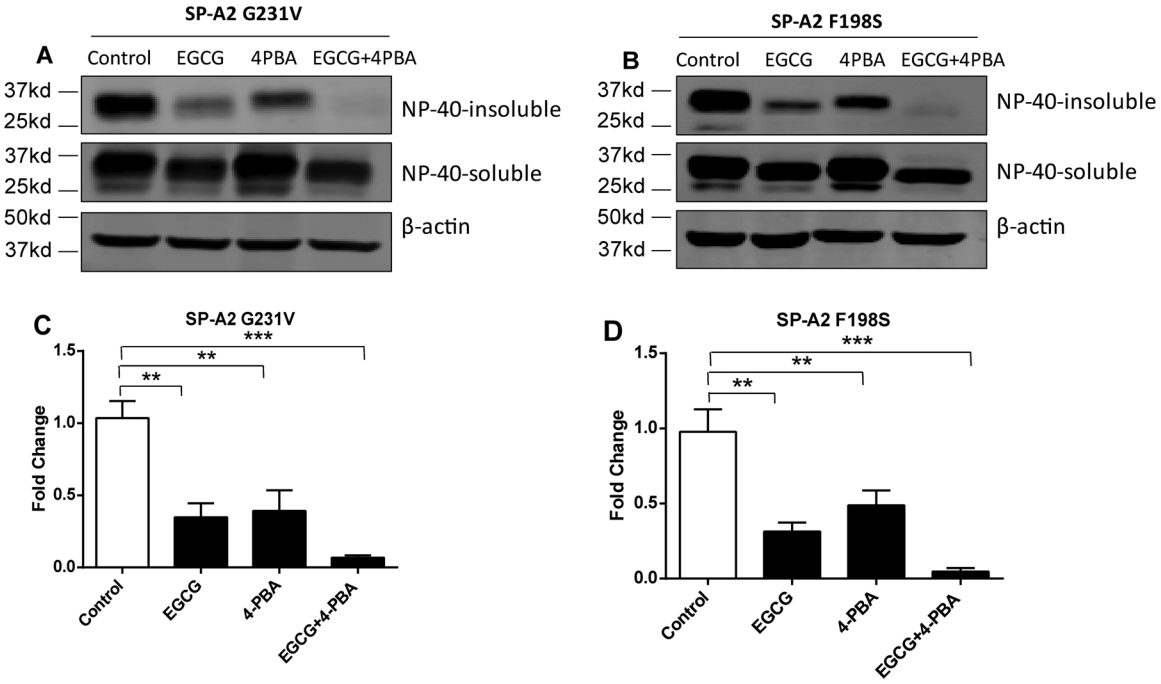

Fig. 4. Combined treatment with EGCG and 4-PBA completely blocked mutant SP-A2 aggregate formation. One day after being transfected with G231V plasmid (A) and F198S plasmid (B), CHO-K1 cells were treated with either EGCG, 4-PBA, or the combination of both for 48 h. Then, both NP-40-insoluble and -soluble fractions were examined by immunoblot analysis. Quantitative results of NP-40-insoluble aggregates from G231V (C) or F198S (D) variants are shown. Data are shown as the mean ± S.D. for at least three independent transfection experiments. **P < 0.01, ***P < 0.001 by one-way ANOVA.

treatment decreased dimer/trimer assembly of mutant protein but not wild-type, suggesting that EGCG-induced reduction in wild-type protein secretion might not due to impaired dimer/trimer formation (Fig. 5C). Meanwhile, EGCG did not alter glycosylation status of mutant SP-A2 as shown in Fig. 5C and D. As we reported previously, SP-A2 mutant proteins are partially glycosylated but they do not undergo sialylation. Therefore, treatment of mutant protein with PNGase F, which removes of all N-linked sugars, resulted in a lower intensity of protein band from cell lysate compared to that without PNGase F treatment. Similar result was obtained by EndoH treatment, which removes high mannose sugars from protein. Meanwhile, neuraminidase and O-glycosidase, which removed sialic acid and O-linked sugars, respectively, did not alter the molecular weight of mutant proteins in cell lysate either. Previous study suggested that expression of G231V and F198S variants upregulated ER stress marker, such as Bip (GRP78), which resides in ER and could prevent non-native polypeptide aggregation. Therefore, we investigated whether EGCG could cause mutant SP-A2 aggregation by increasing Bip protein expression level. However, EGCG treatment did not alter Bip expression as observed in CHO-K1 cells transiently transfected with wild-type or mutant SP-A2 (Fig. 5E and F), indicating that EGCG-mediated reduction of NP-40-insoluble aggregates of SP-A2 mutant protein may not involve Bip-mediated protein refolding pathway.

3.6. EGCG reduced SP-A2 protein stability

To further explore the potential molecular mechanism of EGCG-induced reduction in aggregate formation of mutant SP-A2, we examined the effects of EGCG on mutant protein stability by performing a chymotrypsin proteolysis assay. As shown in Fig. 6A, SP-A2 G231V protein is sensitive to α-chymotrypsin-mediated digestion in a time-dependent manner and the $t_{1/2}$ was calculated to be 6.93 ± 1.02 min (Table. 1). Interestingly, EGCG treatment enhanced the sensitivity of G231V protein to chymotrypsin digestion, as the $t_{1/2}$ decreased to 2.47 ± 0.67 min (Fig. 6A and B, Table 1). Similarly, EGCG significantly decreased the sensitivity of SP-A2 F198S protein to chymotrypsin digestion, as the $t_{1/2}$ changed from 4.97 ± 0.33 min to 1.17 ± 0.15 min (Fig. 6C and D, Table 1). Unexpectedly, we found that EGCG treatment increased wild-type SP-A2 sensitivity to chymotrypsin. The calculated $t_{1/2}$ of wild-type SP-A2 changed to 25.53 ± 1.41 min in presence of EGCG compared to 33.53 ± 2.72 min without EGCG treatment (Fig. 6E and F, Table 1).

3.7. EGCG reduced the formation of mutant SP-A2 aggregates via proteasomal pathway

As previously reported, SP-A2 mutant protein mainly degraded via proteasomal pathway. Therefore, we speculated that EGCG might also degrade the aggregates of mutant SP-A2 via this pathway. Indeed, without EGCG treatment, NP-40-insoluble aggregates formed from both mutant SP-A2 variants accumulated in cells in the presence of MG132, an inhibitor of proteasome. This result indicated that the aggregates of mutant protein are partially degraded via proteasomal pathway in cells under normal conditions, but the rest of the aggregates still accumulated in cells. When treated with EGCG, these aggregates are further degraded via proteasomal pathway, as the results (Fig. 7A and C) showed that EGCG-mediated reduction in aggregation of mutant protein (Fig. 7A and C, lane 2) is reversed in presence of MG132 (Fig. 7A and C, lane 4). Moreover, MG132 showed similar effects on the NP-40 soluble fraction in the cell lysate (Fig. 7B and D).These data indicated that EGCG might activate and promote the proteasomal pathway so as to facilitate degradation of more mutant protein aggregates. At the same time, we also determined the role of autophagy and lysosomal pathways in EGCG-mediated reduction of mutant protein using specific inhibitors, 3MA and leupeptin, respectively. Both inhibitors did not alter the levels of NP-40-insoluble (Fig. 7E, G, I, K) or -soluble fraction (cell lysate) (Fig. 7F, H, J, L) of mutant proteins. These results confirmed that the aggregates of mutant SP-A2 could be degraded via the proteasomal pathway but not the autophagy or lysosomal pathway, and EGCG could further promote the proteasomal pathway to reduce the formation of mutant SP-A2 aggregates in CHO-K1 cells. We also confirmed that wild-type SP-A2 did not degrade via any of these three pathways both in cell lysate and medium (Fig. 8A–F).

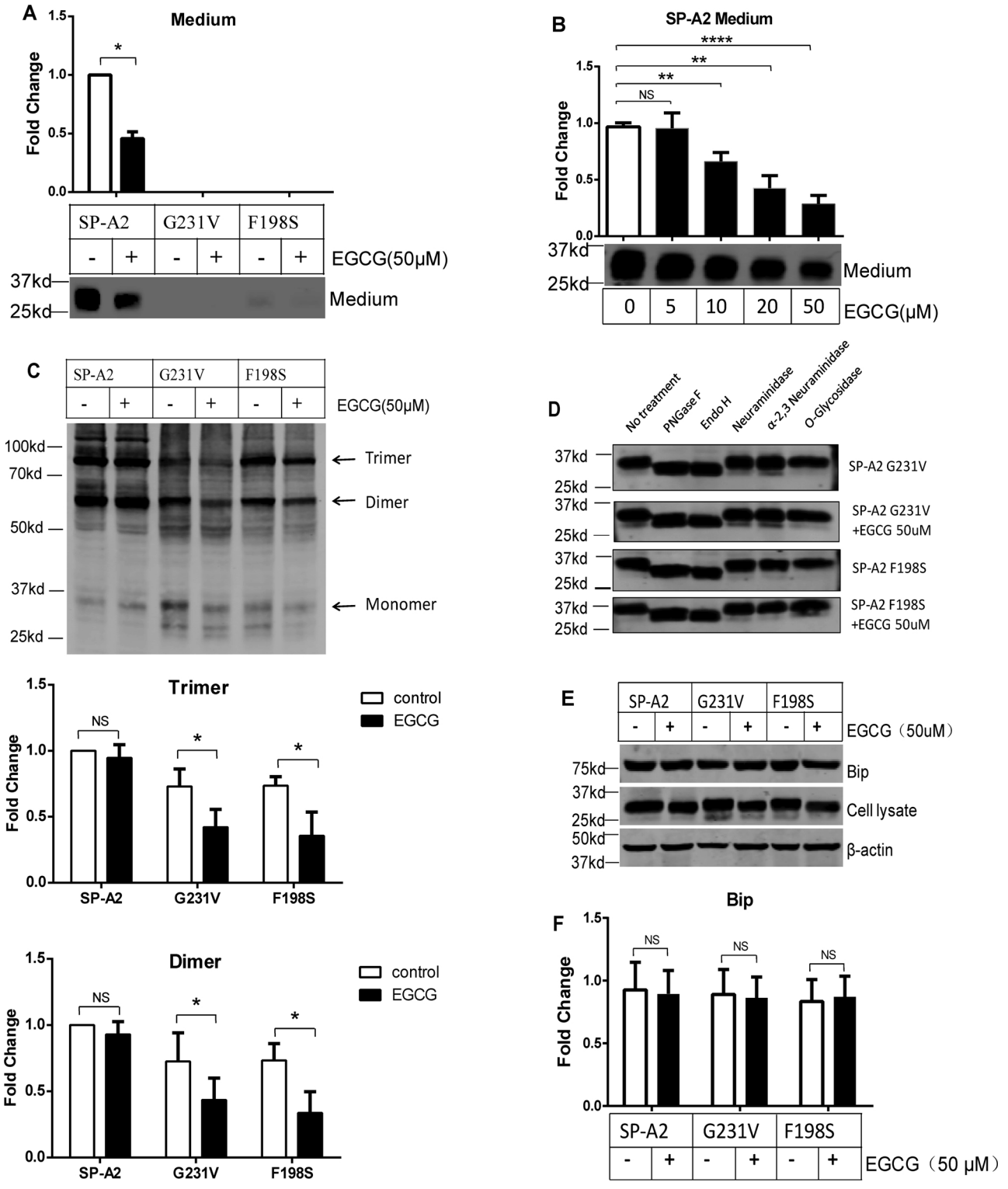

Fig. 5. EGCG did not promote mutant protein secretion and did not upregulate the expression of Bip. CHO-K1 cells were transiently transfected with SP-A2 wild-type, G231V, or F198S variants. Twenty-four hours later, the medium was changed, and the cells were incubated with or without EGCG for 48 h. The medium was collected, and secretory protein was detected by immunoblotting (* $P < 0.05$ by unpaired *t*-test after ANOVA) (A). The secretion of wild-type SP-A2 in the medium was EGCG dose-dependent (** $P < 0.01$, **** $P < 0.0001$ by unpaired *t*-test after one-way ANOVA; NS, no significance.) (B). The cell lysates were subjected to non-reducing gel (C) to identify SP-A2 dimer/trimer (* $P < 0.05$ by unpaired *t*-test after one-way ANOVA; NS, no significance.). (D) Glycosylation modifications of mutant SP-A2 were analyzed by PNGase F, Endo H, neuraminidase, α-2,3-neuraminidase, and *O*-glycosidase treatment. (E–F) Bip protein expression was also detected and quantified in cell lysate in presence or absence of EGCG (unpaired *t*-test after one-way ANOVA; NS, no significance.). Data are shown as the mean ± S.D. for at least three independent transfection experiments (*$P < 0.05$).

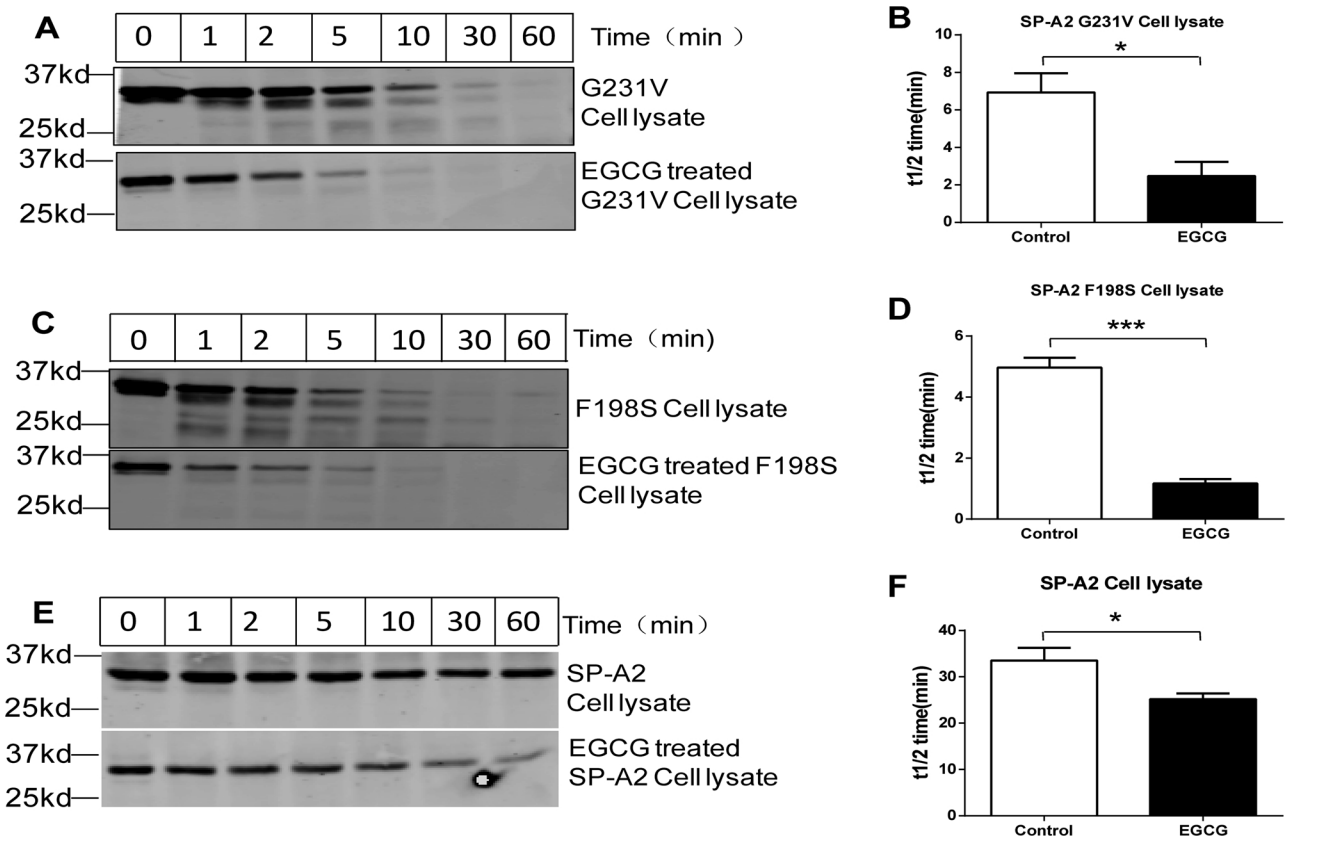

Fig. 6. EGCG caused SP-A2 protein instability. CHO-K1 cells that had been transfected with SP-A2 wild-type, G231V, or F198S variants were treated with EGCG for 48 h and equal amounts of total protein from cell lysate were distributed to different reaction tubes followed by treatment with chymotrypsin for different time periods ranging from 0 to 60 min (0, 1, 2, 5, 10, 30, 60 min) at a mass ratio of 1:50. Then, they were subjected to SDS-PAGE followed by immunoblot analysis using a monoclonal antibody that recognized the V-5 epitope (A,C,E).The half-life of each sample was calculated, and the result for each plasmid was presented as above (B,D,F). Data are shown as the mean ± S.D. for at least three independent transfection experiments (*P < 0.05, ***P < 0.001 by unpaired *t*-test).

Table 1
The $t_{1/2}$ (minutes) of SP-A2 protein digested with chymotrypsin.

SP-A2	$t_{1/2}$(min)
SP-A2 wild-type	33.53 ± 2.72
EGCG treated SP-A2 wild-type	25.53 ± 1.41
SP-A2 G231V	6.93 ± 1.02
EGCG treated SP-A2 G231V	2.47 ± 0.76
SP-A2 F198S	4.97 ± 0.33
EGCG treated SP-A2 F198S	1.17 ± 0.15

4. Discussion

Previous studies have demonstrated that SP-A2 mutations at carbohydrate recognition domain (CRD), G231V and F198S, lead to protein misfolding, leading to formation of NP-40-insoluble aggregates and protein secretion deficiency. In this study, we showed that EGCG, a naturally-occurring small molecule, which was extracted from green tea, significantly decreased mutant SP-A2 protein aggregation, as well as wild-type SP-A2 aggregation when co-expressed with G231V variant. EGCG in conjugation with 4-PBA could completely eliminate these aggregates. These effects of EGCG might be caused by reducing protein stability and enhancing proteasomal pathway-mediated protein degradation.

Accumulating evidence has suggested that some IPF patients with genetic mutations exhibited the toxic effects of misfolded protein aggregates, which usually induced ER stress and caused functional disparity in alveolar type II cells (Lawson et al., 2011; Maitra et al., 2010). Therefore, elucidating the effects and potential mechanisms of misfolded protein aggregation is important, which might help to develop novel therapies against IPF.

It has been previously shown that several diseases arise due to formation of aggregates of misfolded proteins, which is caused by mutations in genome or environment insults, especially in cases of neurodegenerative diseases, such as Alzheimer's disease (AD), Down syndrome (DS), and Parkinson's disease (PD) (Wang et al., 2014). Protein misfolding and assembling into fibrillar aggregates, as well as the formation of amyloid fibrils is one of the hallmarks of these diseases (Mannini et al., 2014). It has been previously reported that EGCG could directly bind to the aggregates of misfolded protein and transform their conformation to redirect amyloidogenic polypeptides into unstructured, off-pathway oligomers (Ehrnhoefer et al., 2006; Bieschke et al., 2010; Ehrnhoefer et al., 2008). This coincides with our result that EGCG decreased aggregates of mutant SP-A2 proteins.

ER is a specialized organelle for synthesis and folding of secretory, membrane and a number of organelle-targeted proteins, and refolding of misfolded proteins in eukaryotic cells (Grek and Townsend, 2014). Our data showed that NP-40-insoluble aggregates of SP-A2 mutant protein would accumulate within the ER (Song et al., 2012). Misfolded protein in the cells will be either refolded or degraded through ubiquitin-proteasome, lysosome, or autophagy pathway (Rubinsztein, 2006; Zhao et al., 2007). Here, we have proved that mutant SP-A2 could be partially degraded through the proteasomal pathway, but not lysosomal or autophagy pathway in CHO-K1 cells. Furthermore, EGCG might reduce mutant protein aggregates by enhancing the proteasomal activity. This result is consistent with earlier studies showing EGCG can reduce polycomb protein level via a proteasome pathway in skin cancer cells (Choudhury et al., 2011). Moreover, EGCG can contribute to the degradation of DNMT3A

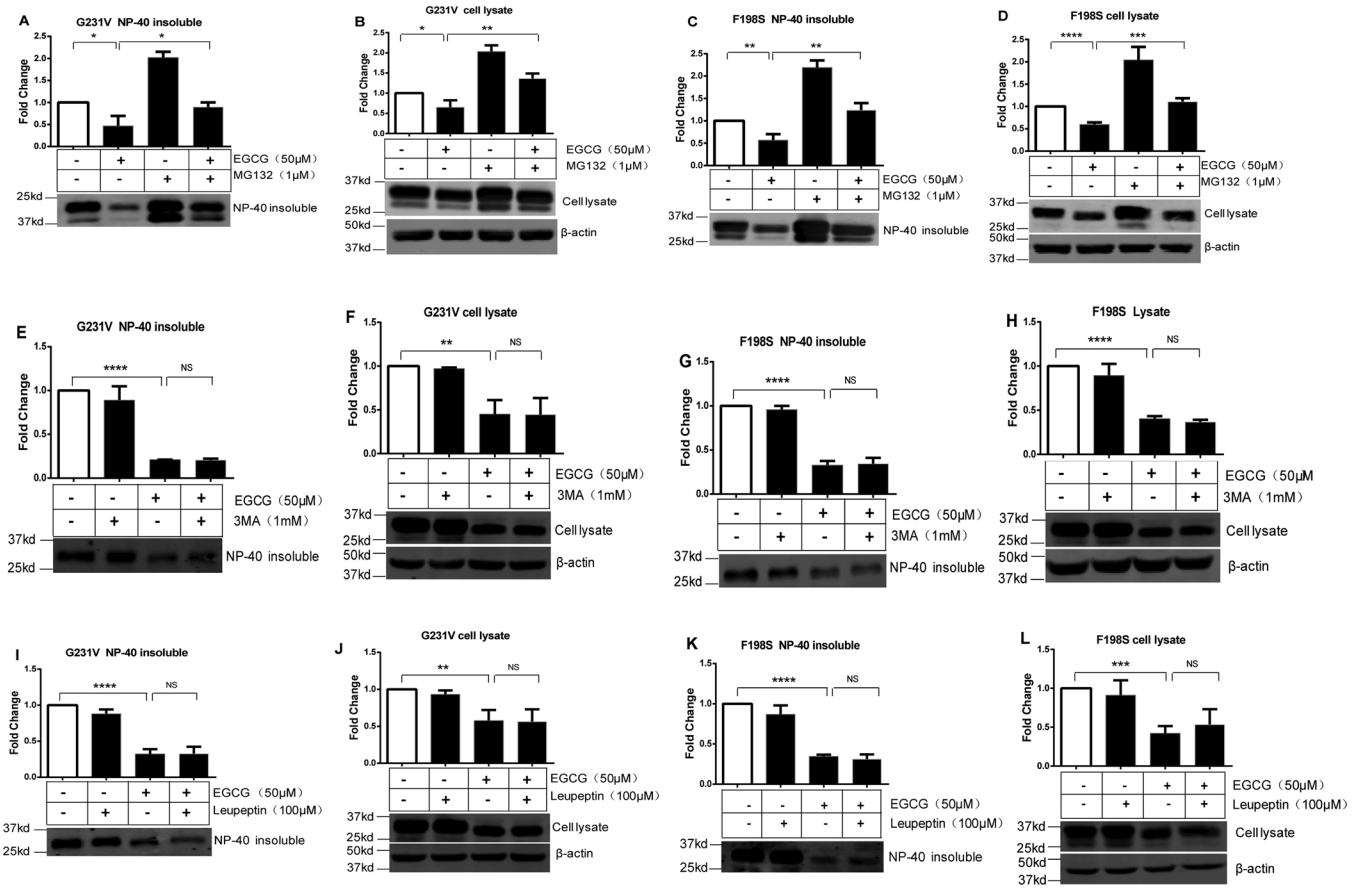

Fig. 7. Reduction of EGCG-induced mutant SP-A2 aggregation via the proteasomal degradation pathway. CHO-K1 cells were transiently transfected with SP-A2 G231V and F198S plasmids. Twenty four hours later, the medium was changed and the cells were co-treated with EGCG and either MG-132(A–D) or 3-MA (E–H), or leupeptin (I–L) for 24 h. Then the NP-40-insoluble aggregates and cell lysates were analyzed by SDS-PAGE and western blotting. The amount of each SP-A2 variant was normalized to its expression in the absence of inhibitors and EGCG. Data are shown as the mean ± S.D. for at least three independent transfection experiments. *P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 by unpaired *t*-test after one-way ANOVA; NS, no significance.

and HDAC3 via the E3 ubiquitin ligase in human colon cancer cells (Moseley et al., 2013). In addition, previous data showed that EGCG could promote the rapid protein kinase C- and proteasome-mediated degradation of Bad (Kalfon et al., 2007). However, EGCG is also considered to be an inhibitor of proteasome due to its suppression of chymotrypsin-like and caspase-like activity of proteasome (Sukhthankar et al., 2008; Nam et al., 2001; de Bettignies and Coux, 2010; Chen et al., 2005). This inconsistence might due to the different type of proteins or cells. In addition, some evidence has also suggested that EGCG could recruit misfolded protein aggregates and then enhance autophagy–lysosomal pathway for their degradation (Li et al., 2011; Giordano et al., 2014). But our results showed that neither autophagy inhibitor, 3MA, nor lysosome inhibitor, leupeptin, reversed the reduction of misfolded protein aggregates by EGCG. In this case, we might add this novel anti-protein aggregation function to EGCG, which has been well described to be anti-oxidant and anti-inflammatory properties.

Protein glycosylation, including sialylation, is one of the important post-translational modifications, which plays a crucial role in maintaining protein stability (Wujek et al., 2004). Coincidentally, SP-A2 variants G231V and F198S are do not undergo sialylation which render them to be more vulnerable to chymotrypsin proteolytic digestions as reported previously (Maitra et al., 2010). In this study, although EGCG increased the sensitivity of SP-A2 protein to chymotrypsin-mediated proteolysis, it did not alter the degree of glycosylation of SP-A2. Therefore, EGCG-mediated instability of SP-A2 protein may not be due to glycosylation deficiency. Moreover, activation of trypsin-like activity of 20S proteasome could be mediated by EGCG. According to our results, protein instability caused by EGCG might promote degradation of SP-A2 mutant protein via proteasomal pathway and reduction in protein aggregation.

Although both EGCG and 4PBA could reduce mutant protein aggregation, EGCG did not reverse the secretion defects of mutant proteins, exposure to only 4PBA did alter these effects, suggesting that the potential mechanisms of aggregate elimination might be different. 4PBA might regulate chaperones (such as Bip) to help misfolded mutant protein refold, and then, promote protein secretion (data not shown), while EGCG might not (Fig. 5E). Probably, EGCG might enhance the proteasomal pathway to reduce the protein stability and lead to degradation of protein aggregates. Unexpectedly, the secretion of wild-type SP-A2 was reduced with EGCG treatment. The association of SP-A2 secretion with the protein assembly of dimer/trimer was validated under non-reducing conditions. However, there is no difference in wild-type SP-A2 dimer/trimer formation with or without EGCG treatment (Fig. 5C). We speculated that EGCG present in the medium might directly reduce the stability and cause partial degradation of wild-type SP-A2, and showed that SP-A2 protein level in medium significantly reduced in presence of EGCG *in vitro* (Fig. 5B).

5. Conclusion

In summary, this study demonstrated that EGCG significantly reduced aggregation of mutant SP-A2 protein (G231V, F198S), associated with pulmonary fibrosis, by decreasing SP-A2 protein stability and promoting its degradation by proteasome-dependent pathway. It was suggested that EGCG might be a remodeling reagent of SP-A2 aggregates and a potential target for pulmonary fibrosis characterized by dysfunction of protein folding.

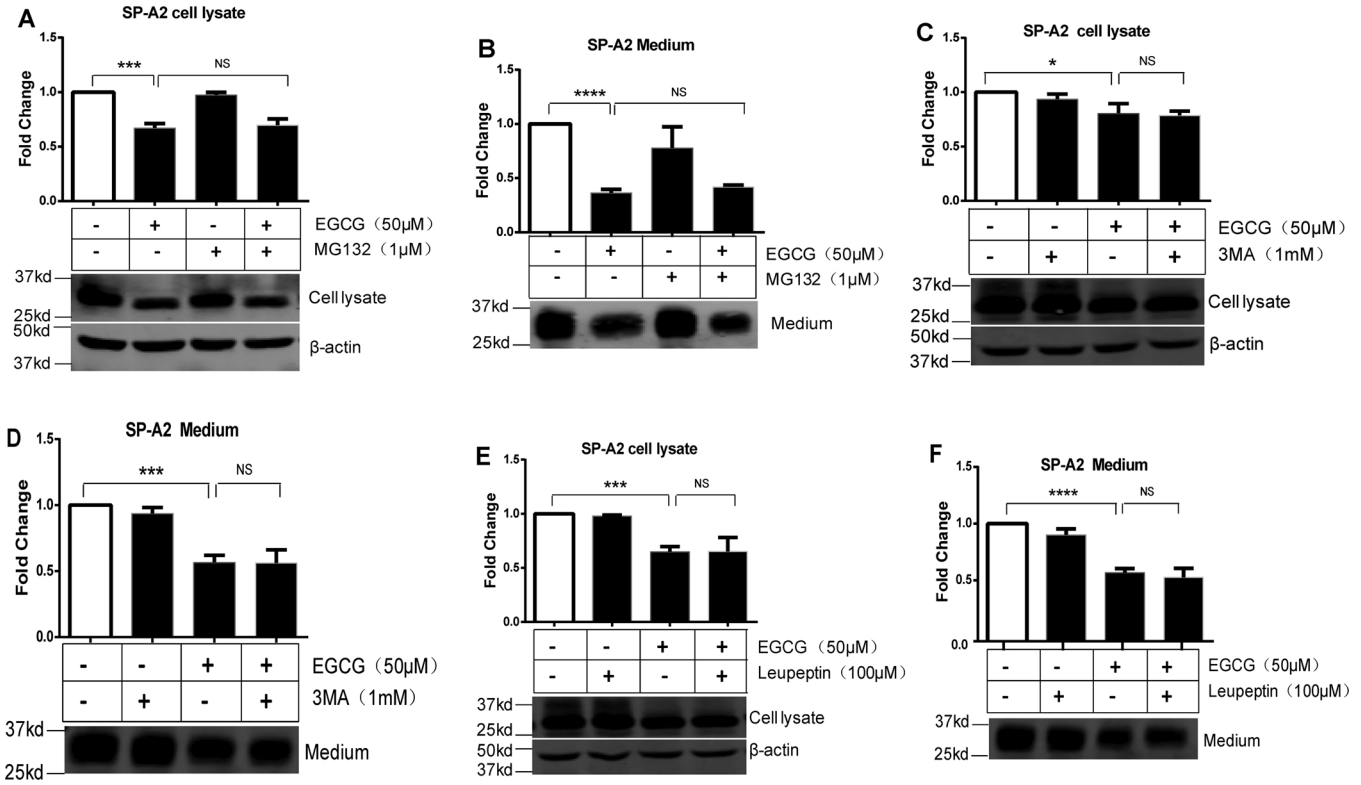

Fig. 8. MG132, 3MA, and leupeptin did not alter the wild-type SP-A2 expression after EGCG treatment. After being transfected with wild-type SP-A2 plasmids for 24 h, CHO-K1 cells were co-treated with either EGCG and MG132, EGCG and 3MA, or EGCG and leupeptin for additional 24 h. Then the cell lysates and media were subjected to SDS-PAGE and immunoblot analysis using V-5 tagged antibody. EGCG in conjugation with MG132 (A–B), or 3MA (C–D), or leupeptin (E–F) did not alter wide-type SP-A2 protein expression as observed in cell lysate (A, C, E) and medium (B, D, F). Data are shown as the mean ± S.D. for at least three independent transfection experiments. *P < 0.05, *** P < 0.001, **** P < 0.0001 by unpaired *t*-test after one-way ANOVA; NS, no significance.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105612.

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