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Chemical chaperone 4-phenylbutyric acid alleviates the aggregation of human familial pulmonary fibrosis-related mutant SP-A2 protein in part through effects on GRP78

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ABSTRACT

G231V and F198S mutations in surfactant protein A2 (SP-A2) are associated with familial pulmonary fibrosis. These mutations cause defects in dimer/trimer assembly, trafficking, and secretion, as well as cause mutant protein aggregation. We investigated the effects and mechanisms of chemical chaperones on the cellular and biochemical properties of mutant SP-A2. Chemical chaperones, including 4-phenyl butyric acid (4-PBA), could enhance secretion and decrease intracellular aggregation of mutant SP-A2 in a dose-dependent manner. Interestingly, increased levels of aggregated mutant SP-A2, resulting from MG-132-mediated proteasome inhibition, could also be alleviated by 4-PBA. 4-PBA treatment reduced the degradation of mutant SP-A2 to chymotrypsin digestion in CHO-K1 cells and up-regulated GRP78 (BiP) expression. Overexpression of GRP78 in SP-A2 G231V- or F198S-expressing cells reduced, whereas shRNA-mediated knockdown of GRP78 enhanced aggregation of mutant SP-A2, suggesting that GRP78 regulates aggregation of mutant SP-A2. Together, these data indicate chemical chaperone 4-PBA and upregulation of GRP78 can alleviate aggregation to stabilize and facilitate secretion of mutant SP-A2. The up-regulation expression of GRP78 might partially contribute to the aggregate-alleviating effect of 4-PBA.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause that primarily occurs in older adults and lacks effective medical therapy [1]. Although aberrant responses to repeated injury of the alveolar epithelium, by exogenous or endogenous stimuli, may contribute to the pathogenesis of the disease, recent evidence suggests that genetic predisposition is another important pathogenetic mechanism [2]. Genome-wide association studies (GWAS) and familial IPF studies have led to identification of multiple susceptibility genes or loci for pulmonary fibrosis, such as rare mutations in TERT, TERC, SFTPC and polymorphic variants in MUC5B [3–7]. Recently, two mutations in the carbohydrate recognition domain (CRD) of surfactant protein A2 (SP-A2) gene (G231V and F198S) have also reported to be associated with familial pulmonary fibrosis [8]. Intriguingly, *in vitro* studies have shown that SP-A2 G231V and F198S mutations cause protein instability and ER stress in epithelial cells [9]. Our prior study also revealed that SP-A2 G231V and F198S mutations cause protein retention in the ER and impair dimer/trimer assembly, which results in deficiency in protein sialylation and secretion when expressed in cells [10].

SP-A, a member of the collectin family, is the most abundant pulmonary surfactant protein and plays a critical role in lung innate immunity and surfactant-related functions [11]. Two functional genes, SP-A1 (SFTPA1) and SP-A2 (SFTPA2), are present in humans and primates (rodents have one gene) and show structural similarity, but functional divergence [12,13]. The primary structure of mature SP-A is highly conserved among different mammals and consists of four structural domains, including an N-terminal non-collagenous domain (7–10 amino acids), a 79-residue collagen-like domain, a 35 amino acid-α-

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helical neck domain, and a 115-residue C-terminal globular-like CRD. Usually, SP-A polypeptide chains can assemble into homo- or hetero-trimers and then six trimers can further build up to the octadecamers. SP-A also undergoes glycosylation [14], acetylation [15], and hydroxylation [16,17] modification. Although both genes are expressed primarily in lung alveolar type II cells, SP-A2 seems more biologically active than SP-A1 in most *in vitro* assays investigated [13].

Additional genetic mutations of SP-A2 in familial and sporadic idiopathic interstitial pneumonias were identified in other studies [18]. Genetic polymorphisms and altered protein expression levels of SP-A have also been linked to other human lung diseases, such as respiratory distress syndrome, and allergic bronchopulmonary aspergillosis [19–21]. The changes of SP-A expression levels are found in the lungs of patients with cystic fibrosis [22,23] and respiratory distress syndrome [24], as well as hypersensitivity pneumonitis [25]. Therefore, to further study the effects of SP-A2 mutations on protein biochemical properties and cell functions will not only help to elucidate the molecular pathogenesis of IPF and related lung diseases, but also to develop potential diagnostic and therapeutic approaches.

A series of small molecules, named chemical chaperones, possess chaperone-like activity and can stabilize mutant proteins, facilitate proper folding of proteins, and suppress aggregation. One of the well-known chemical chaperones, 4-phenylbutyric acid (4-PBA), has been shown to prevent the misfolding and mislocalization of proteins, such as the cystic fibrosis transmembrane conductance regulator (CFTR) [26] and α_1-antitrypsin [27]. 4-PBA also alleviates intracellular aggregate formation by a variety of mutant proteins, for example HFE C282Y aggregates [28,29]. However, how chemical chaperones, such as 4-PBA, affect mutant SP-A2 is still largely unknown. To address this question will clarify the mechanism of genetic diseases as well as develop the chemical chaperone as a potential therapeutic method for these diseases.

We used CHO-K1 and A549 cells, transiently expressing wild-type or mutant SP-A2, as a model to investigate how molecular chaperones affect mutant SP-A2 protein processing, secretion and potential molecular interactions. Here we reveal that several chemical chaperones, including 4-PBA, not only reduce NP-40-insoluble aggregates, but also rescue protein secretion of mutant SP-A2. Furthermore, 4-PBA treatment increases the resistance of mutant SP-A2 to α-chymotrypsin digestion, and blocks abnormal retention in the endoplasmic reticulum (ER). Interestingly, wild-type SP-A2 and SP-A2 G231V, but not SP-A2 F198S, can co-precipitate in aggregates. This dominant-negative effect of G231V is reversed by 4-PBA treatment. Importantly, we demonstrate that upregulation of GRP78 contributes to reduced aggregation of mutant SP-A2 protein, which might be one of mechanisms for 4-PBA-alleviated aggregation of mutant SP-A2 protein.

2. Experimental procedures

2.1. Materials

Culture medium and fetal bovine serum (FBS) were obtained from HyClone (Thermo Scientific, Rockford, IL). Protease inhibitor cocktail tablets were purchased from Roche Applied Science. Anti-V5 mouse monoclonal Ab (R960-25) was obtained from Invitrogen, and anti-calreticulin (H-170) and anti-GRP78 rabbit polyclonal antibody (SC13968) from Santa Cruz Biotechnology (Santa Cruz, CA); IRDye 800CW-conjugated goat anti-mouse secondary antibody was from LI-COR Biosciences. HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from Southern Biotech (Birmingham, AL). All other chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise indicated.

For transient expression in mammalian cells, the pcDNA3.0 vector was used. All PCRs for cloning utilized high-fidelity DNA polymerases and all subclones were confirmed by sequence analysis. Two partial IMAGE cDNA clones, 5184888 and 841707 (Invitrogen) were used to construct a full-length human SP-A2 cDNA. The N- and C-terminal halves were PCR amplified, combined and again PCR amplified. Full-length SP-A2 cDNA was cloned into the pGEM-T Easy vector, then digested with *Eco*RI and subcloned into pcDNA3.0. An in-frame V5 epitope tag was constructed, after the glutamic acid at amino acid 21, by primer extension mutagenesis and zipper PCR. The DNA sequence of the V5-tag was 5′-GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG-3′. Site-directed mutagenesis (QuickChange, Stratagene) was utilized so that the DNA sequence of wild-type SP-A2 clones exactly matched NM_006926.2.

2.2. Cell culture and transfection

CHO-K1 cells (CCL-61) and A549 cells (CCL-185) were purchased from the American Type Culture Collection and maintained in DMEM/F12 with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin, and incubated at 37 °C with 5% CO_2. Cells were transiently transfected with different expression constructs using FuGENE HD Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol.

2.3. Generation of stable cell lines

Stable CHO-K1 clones were obtained by standard procedures. Briefly, cells with 80% confluence in 6 cm dishes were transfected with the pcDNA3.0 vector or SP-A2 variants plasmids. After selection with 0.5 μg/ml of neomycin, resistant cells were cloned by limiting-dilution. The expression of V5-tagged SP-A2 variants was confirmed by western blotting.

2.4. 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 aliquots were subjected to electrophoresis on 10% SDS-PAGE Bio-Rad minigels and transferred to nitrocellulose (NC) Protran membranes (Whatman, Dassel, Germany). Blots were incubated for 1 h at RT in blocking buffer (5% dried milk in TBST (150 mM NaCl, 10 mM Tris, pH 8.0, 0.1% Tween-20)), and with primary antibody overnight at a dilution of 1:10,000 (anti-V5 antibody) in blocking buffer. After washing 4 times in TBST for 5 min each, blots were incubated with secondary antibody (1:20,000 in blocking buffer) for 1 h at RT, washed 4 times in TBST for 5 min each, and visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL) and exposure to Kodak film.

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

CHO-K1 or A549 cells in 6-well plates were transiently transfected with V5-tagged SP-A2 constructs (1 μg/well) using the FuGENE HD transfection reagent according the manufacturer's procedure. At 72 h post-transfection, cells were washed once with 2 ml of ice-cold PBS, harvested in ice-cold 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 rocked at 4 °C for 30 min. Cell lysates were sedimented at 16,000*g* for 10 min at 4 °C. The supernatants were saved as the NP-40-soluble fraction. Pellets were washed once with lysis buffer and solubilized with 2 × SDS buffer (125 mM Tris·HCl, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue) and saved as the NP-40-insoluble fraction [30].

2.6. Chymotrypsin limited proteolysis assay

After transient transfection for 72 h, CHO-K1 or A549 cells in each 35 mm dish were lysed in 250 μl lysis buffer (100 mM NaCl, 50 mM