# THE UNFOLDED PROTEIN RESPONSE CONTROLS ER STRESS-INDUCED APOPTOSIS OF LUNG EPITHELIAL CELLS THROUGH ANGIOTENSIN GENERATION

By

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**ABSTRACT**

**THE UNFOLDED PROTEIN RESPONSE CONTROLS ER STRESS-INDUCED APOPTOSIS OF LUNG EPITHELIAL CELLS THROUGH ANGIOTENSIN GENERATION**

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Apoptosis of alveolar epithelial cells (AECs) is a critical event in the pathogenesis of pulmonary fibrosis. Recent studies showed that the precursor SP-C contains a BRICHOS domain which has chaperone-like properties that protect the peptide from aggregation. SP-C BRICHOS mutants have been shown to induce endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR), leading to cell death and subsequent lung fibrosis. Therefore, understanding the pathogenesis of pulmonary fibrosis requires us to understand the regulation of AEC apoptosis. Previous studies from our laboratory have shown that angiotensin-converting enzyme 2 (ACE-2) plays a protective role against fibrosis by degrading the pro-apoptotic peptide angiotensin II (ANGII) to generate the anti-apoptotic peptide angiotensin 1-7 (ANG1-7), which inhibits AEC apoptosis. We also showed that the generation of ANGII, which is mediated by cathepsin D, is important for the progression of lung fibrosis.

Our laboratory was the first to show that ER stress-induced apoptosis of AECs is regulated by the autocrine ANGII/ANG1-7 system. I examined the role of the angiotensin system in response to ER stress by using the proteasome inhibitor MG132 as a chemical inducer of human AECs. MG132-induced ER stress upregulated ANGII-generating enzyme cathepsin D and downregulated ANGII-degrading enzyme ACE-2 in human epithelial cells (Chapter 2). Human AECs in response to ER stress were assessed for apoptosis by measuring mitochondrial

function, caspase activation and nuclear fragmentation. It was demonstrated that ER stress-induced apoptosis of AECs was significantly prevented by the ANG receptor blocker, saralasin or drastically inhibited by ANG1-7. Moreover, blockade of ANG1-7 by the specific *mas* antagonist, A779 could enhance the ER stress-induced apoptosis of AECs. These data suggest that ER stress-induced apoptosis of AECs might be regulated by the autocrine ANGII/ANG1-7 system, and modulation of the ACE-2/ANG1-7/mas axis could provide support for new therapeutic treatment of fibrotic diseases.

It was hypothesized that ER stress-induced apoptosis of human AECs might be mediated by the unfolded protein response (UPR) via the autocrine ANGII/ANG1-7 system (Chapter 3). A549 cells were challenged with MG132 or SP-C BRICHOS domain mutant G100S to induce ER stress and UPR activation. MG132 or G100S SP-C mutation activated all 3 canonical pathways of the UPR (IRE1/XBP1, ATF6, and PERK/eIF2α), which led to an increase in cathepsin D or ADAM17/ACE (an ACE-2 ectodomain shedding enzyme) - and eventually caused AEC apoptosis. However, ER stress-induced UPR activation of AECs could be prevented by a chemical chaperone (4-PBA) or by UPR specific blockers (4μ8C, GSK2656157). It also suggested that ATF6 and IRE1 pathways might play important role in regulation of angiotensin system. These data demonstrate that ER stress induces apoptosis of human AECs through mediation of UPR pathways, which in turn regulate the autocrine ANGII/ANG1-7 system.

Collectively, the results in these studies suggest the mechanisms by which ER stress and UPR activation induce apoptosis of human AECs. It provides evidence that manipulation of the angiotensin system might lead to therapeutic strategies to limit lung fibrosis.

*This accomplishment is dedicated to my parents, who have sacrificed everything for me.*
*Without them, I would not be who I am today.*
*Thank you, Mom and Dad, for all of your love and support along the way.*

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## KEY TO ABBREVIATIONS

| | |
|---|---|
| **αSMA** | Alpha-Smooth Muscle Actin |
| **ACE** | Angiotensin Converting Enzyme |
| **ACE-2** | Angiotensin Converting Enzyme-2 |
| **ADAM** | a Distegrin and Metalloproteinase |
| **AEC(s)** | Alveolar Epithelial Cell(s) |
| **AGT** | Angiotensinogen |
| **ANG1-7** | Angiotensin 1-7 |
| **ANGI** | Angiotensin I |
| **ANGII** | Angiotensin II |
| **AT** | Angiotensin Receptor Type (1,2) |
| **ATCC** | American Type Culture Collection |
| **ATF4** | Activating Transcription Factor 4 |
| **ATF6** | Activating Transcription Factor 6 |
| **BCA** | Bicinchoninic Assay |
| **BPD** | Bronchopulmonary Dysplasia |
| **BiP** | Binding immunoglobin Protein |
| **CatD** | Cathepsin D |
| **CF** | Cystic Fibrosis |
| **CFTR** | Cystic Fibrosis Transmembrane Conductance Regulator |
| **CHOP** | CCAAT-enhancer-binding protein homologous protein |

| | |
|---|---|
| **COPD** | Chronic Obstructive Pulmonary Disease |
| **CTL** | Control |
| **DT** | Diphtheria Toxin |
| **ECM** | Extra Cellular Matrix |
| **EMT** | Epithelial-to-Mesenchymal Transition |
| **ER** | Endoplasmic Reticulum |
| **ERAD** | ER-associated protein degradation |
| **FDA** | Food and Drug Administration |
| **FOXO1** | Forkhead Box Protein O1 |
| **GPCR** | G-Protein Coupled Receptor |
| **GRP78** | Glucose-Regulated Protein, 78 kDa |
| **IPF** | Idiopathic Pulmonary Fibrosis |
| **IRE1** | Inositol-Requiring Enzyme 1 |
| **IRS1** | Insulin Receptor Substrate 1 |
| **JNK** | cJun N-Terminal Kinase |
| **kDa** | kilo-Daltons |
| **MAPK** | Mitogen-Activated Protein Kinase |
| **MG** | MG132 |
| **MKP** | Map Kinase Phosphatase-2 |
| **NF-κB** | Nuclear-factor kappa B |
| **PBS** | Phosphate Buffered Saline |
| **PERK** | Protein Kinase RNA-like Endoplasmic Reticulum Kinase |

| | |
|---|---|
| **PI3K** | PhosphoInositide 3-Kinase |
| **RAS** | Renin-Angiotensin System |
| **ROS** | Reactive Oxygen Species |
| **RT-PCR** | Reverse Transcription Polymerase Chain Reaction |
| **SARS-CoV** | Severe Acute Respiratory Syndrome Coronavirus |
| **SP-C** | Surfactant Protein C |
| **TACE** | TNF-α Converting Enzyme |
| **TBS** | Tris-Buffered Saline |
| **TBST** | Tris-Buffered Saline with 0.1% Tween 20 |
| **TLR** | Toll-like Receptor |
| **TNF-α** | Tumor Necrosis Factor-Alpha |
| **WT** | Wild-Type |
| **XBP1** | X-box Binding Protein 1 |

# CHAPTER 1

# INTRODUCTION

## PATHOGENESIS OF PULMONARY FIBROSIS

Pulmonary fibrosis represents a group of interstitial lung disorders caused by an abnormal wound repair process that leads to scar formation, organ malfunction, disruption of gas exchange and respiratory failure (57). Idiopathic pulmonary fibrosis (IPF), the most common form of the interstitial lung disease, is a fatal disease with poor prognosis and limited therapeutic opportunities (32). The median survival after diagnosis of IPF is 2.5 to 3.5 years (72). IPF is more common in men and increases significantly with age (57). Although the mechanisms that result in IPF are still unknown, it was proposed that apoptosis of alveolar epithelial cells (AECs), rather than inflammation, is the critical event underlying the pathogenesis of IPF.

IPF is characterized by progressive fibrosis, excessive matrix deposition leading to destruction of lung architecture and impairment of lung function (72). Over 35 years ago, Wanda Hascheck and Hanspeter Witschi proposed that lung fibrosis was initiated and propagated by microfoci of epithelial damage and that, if unrepaired, upsets the normal epithelial-fibroblast balance – thereby favoring a fibrotic environment (87). In 2001, IPF development and progression were proposed to be initiated and sustained by repeated cycles of AEC injury occurring in the wound healing process of skin, kidney and liver (57). In support of this idea, administration of caspase inhibitor *in vivo* to rats or mice not only prevented the apoptosis of AECs after bleomycin, but also inhibited the subsequent accumulation of lung collagens (95). Moreover, administration of Fas-activating antibodies into mice models induced apoptosis mainly in the lung epithelium that was followed by collagen accumulation (87). Thus,

it is suggested that injury of AECs is strongly involved in the wound healing process of the lung fibrosis.

In injured lung tissues, fibroblasts are activated and differentiate into myofibroblasts – cells with higher fibrotic potential (30). These cells can cause the accumulation of extracellular matrix – the hallmark of the scarring process resulting in pulmonary destruction (30). Many groups suggested that AECs themselves can contribute to the expansion of the populations of fibroblasts and myofibroblasts through the process of epithelial-mesenchymal transition (EMT). EMT is a biologic process that allows a polarized epithelial cell to undergo many biochemical changes that enable it to acquire a mesenchymal cell phenotype, including increased migratory activity, invasiveness, resistance to apoptosis and enhanced production of extra cellular matrix (ECM) components (26). In response to TGF-β, alveolar epithelial cells undergo a loss of epithelial markers (surfactant proteins, keratin 18) and express a high level of mesenchymal markers (N-cadherin, α smooth muscle actin) (30).

Interactions between AECs and fibroblasts have been shown to be important features to the pathogenesis of IPF (10). Biopsies from lungs of IPF patients showed the colocalization of areas of AEC apoptosis and foci of αSMA-positive myofibroblasts, suggesting the interactions between the two cells (72). It is suggested that the injured epithelial cells affect the local fibroblasts through their paracrine signaling pathways. Morishima *et al.* have shown that mechanical injury to epithelial cells upregulated the expression of αSMA as well as type I and type III collagen in cocultured fibroblasts by activating the TGF-β in the ECM (72). Additionally, injured epithelial cells also activated fibroblasts by producing cytokines/growth factors such as

connective tissue growth factor (CTGF), morphogens (sonic hedgehog, Shh), lipid medicators (prostaglandin $E_2$, $PGE_2$). Among those, mediators like TGF-β, CTGF and Shh have been shown to promote the activation of fibroblasts (72). Activated fibroblasts, however, also affect epithelial cells through paracrine signals to induce epithelial apoptosis by activating angiotensin II (ANGII) and reactive oxygen species (ROS).

ANGII, an important component of the angiotensin system, is well-known for its role in blood pressure regulation by its ability to mediate vasoconstriction as well as its proapoptotic effect in fibrotic diseases (72). Studies of lung tissue from IPF patients and bleomycin-challenged mice proved that ANGII is the proapoptotic agent that induces apoptosis of AECs, and that myofibroblats are a key source of ANGII production in fibrosing lung. Mouse models of ANGII type 1 receptor ($AT_1R$) knockout, when challenged with bleomycin, have been shown to have reduced AEC apoptosis and less collagen accumulation (44). Previous studies from our laboratory demonstrated that single-nucleotide polymorphism (SNP) in the promoter region of angiotensinogen (AGT), which increases AGT transcription and later gives rise to ANGII, has been implicated in the rapid progression of lung disease (72).

Type I collagen, a matrix protein in the lung interstitium, is mainly synthesized by activated fibroblasts. Type I collagen causes thickened alveolar walls and have been observed in the fibrotic lungs. Studies from our laboratory have shown that ANGII signaling activates procollagen production in normal lung fibroblasts (85). We also showed that ANGII mediated the synthesis of ECM, CTGF and especially TGF-β. TGF-β, which is activated by epithelial cell integrins, activates the pro-fibrotic activities in fibroblasts, including proliferation,

myofibroblast differentiation and ECM production (72). Additionally, Renzori *et al.* have observed that ANGII stimulated the contraction of collagen gels and αSMA through activation of $AT_1R$ receptor (85). It is suggested that ANGII is an important mediator of the bidirectional interactions between epithelial cells and fibroblasts in pulmonary fibrosis (72). Taken together, understanding of epithelial-fibroblast interactions can provide strategies for potential treatment of fibrotic lung disease.

**APOPTOSIS OF ALVEOLAR EPITHELIAL CELLS**

In the adult lung, the alveolar epithelium is constituted from two morphologically distinct epithelial components: type I and type II alveolar cells (10). Type I cells are large but flatten, while type II cells are cuboidal cells of a size intermediate between smaller endothelial cells and larger macrophages and type I cells (10). Type I AECs, which cover 95% of the alveolar surface area of the peripheral lung, function mainly in gas exchange. Type II AECs are the stem cells of the alveolar epithelium, which can divide and differentiate into type I AECs (27, 89). Mason and Williams were first to describe type II cells as a “defender of the alveolus” (52). Type II AECs reduce surface tension by their capacity to synthesize, secrete and recycle all components of pulmonary surfactant (13, 89). Surfactants have been shown to act as an additional force to direct the fluid flow across the air-blood barrier and maintain the fluid homeostasis (13). Moreover, type II cells contain lamellar bodies, which undergo several steps of transformation that play key role in establishing the surface-active lining layer and cycle of transportation of the surfactant proteins to the macrophages (13).

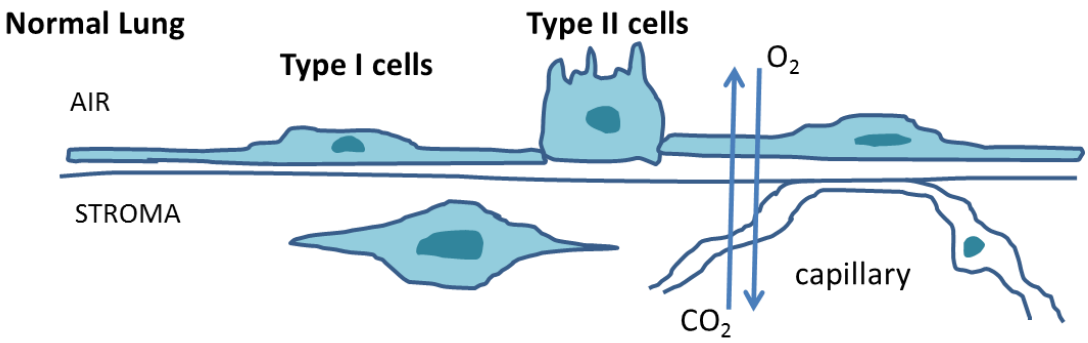


**Figure 1.1. Type 1 and type 2 alveolar epithelial cells.** Type 1 AECs cover about 95% of the alveolar surface and functions as an alveolar barrier. Type 2 AECs reduce the alveolar surface tension by secreting surfactant proteins.

Chronic injury of alveolar epithelial type II cells is now widely accepted as a key event in IPF (18). It was observed by our laboratory that AEC death is the prominent pattern in regions of hyperplastic epithelium covering fibrotic foci of IPF lungs (84). Moreover, apoptosis of type II AECs was found dominantly in mouse models of lung fibrosis exposed to bleomycin. This fibrosis was attenuated with caspase inhibitor or genetic deletion of proapoptotic Bcl2 protein (97). Monogenetic diseases such as Hermansky-Pudlak syndrome (HPS) are characterized by dysfunctional alveolar type II cells, in which mutations in the HPS genes results in the over accumulation of phospholipids due to the dysfunctional lysosome-related organelles of type II cells (57). Sisson and colleagues showed that targeted injury of AEC type II cells by diphtheria toxin (DT) in transgenic mice expressing the DT receptor by a SP-C-driven promoter resulted in pulmonary fibrosis (62). Recently, it has been discovered that familial forms of IPF are caused by mutations in the surfactant protein C (SP-C) leads to ER stress and apoptosis of AECs (79, 80). SP-C is exclusively secreted by type II AECs.

Apoptosis, also known as a programmed cell death, is a physiological event that plays critical role in the development and homeostasis of the cells. However, inappropriate apoptosis (either too little or too much) has been reported as a factor in numerous human diseases including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer (11). Apoptosis of AECs has long been considered as an essential feature of IPF (75), as it is persistently found in lung tissues from patients with IPF (68, 84) and in mouse models of lung fibrosis (44). Experimental studies have demonstrated that inhibition of apoptosis by either caspase inhibitors (20, 94, 95) or deletion of genes critical to apoptosis (5) could abrogate the fibrotic response, thus preventing lung injury (83).

Apoptosis is a multi-pathway cell death program that is tightly regulated at multiple points (23). There are two main apoptotic pathways: the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway. However, these two pathways are linked and molecules in one pathway can affect the other (11). In both pathways, cysteine aspartyl-specific proteases (caspases) are activated and cleave substrates, leading to the biochemical and morphological changes which become characteristics of apoptosis (23). During apoptosis, cells are smaller in size, the cytoplasms are dense and the organelles are more tightly packed (11). Additionally, extensive plasma membrane blebbing occurs followed by formation of apoptotic bodies, and the nuclear DNA breaks up into fragments (11). This process alters the cell surface and expresses the ligands for phagocytic cell receptors that cause the dying cell to be phagocytosed, either by neighboring cells or by macrophages.

**LOCAL RENIN-ANGIOTENSIN SYSTEM IN LUNG FUNCTIONS**

The renin-angiotensin system (RAS) plays a critical role in cardiovascular and renal functions by its abilities to maintain blood pressure homeostasis as well as electrolyte balance (24). Dysfunction of RAS has been associated with the pathogenesis of a variety of diseases, including heart, kidney, liver and lung. The local angiotensin system of lung AECs consists of angiotensinogen (AGT), an aspartyl protease such as renin or cathepsin D, angiotensin-converting enzyme (ACE), angiotensin II (ANGII), angiotensin type I and type II receptors ($AT_1R$ and $AT_2R$), angiotensin-converting enzyme 2 (ACE-2), angiotensin 1-7 (ANG1-7) and its receptor m*as* (86).

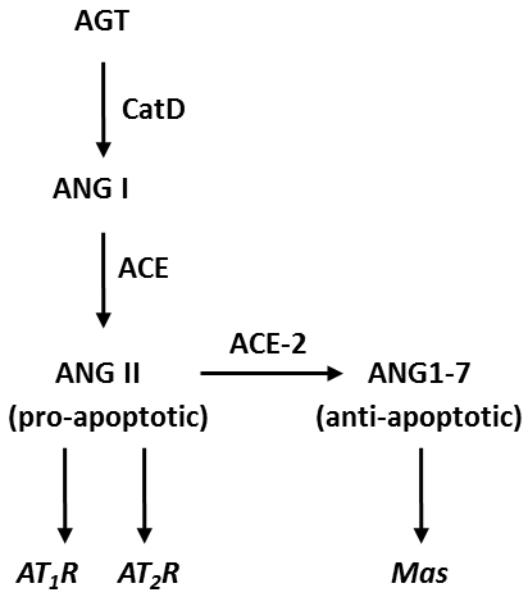


**Figure 1.2. Local angiotensin system in alveolar epithelial cells.** Angiotensinogen (AGT) is cleaved by cathepsin D (CatD) to form angiotensin I (ANGI). ANGI is then further cleaved by angiotensin-converting enzyme (ACE), a dipeptidyl carboxypeptidase, to generate angiotensin II (ANGII). Angiotensin-converting enzyme 2 (ACE-2) cleaves ANGII to form angiotensin 1-7 (ANG1-7). *$AT_1R$* and *$AT_2R$* are two receptors of ANGII, whereas *Mas* is the receptor of ANG1-7.

**Angiotensinogen (AGT).** AGT is a high molecular weight protein and is a precursor of ANGII. Transcriptional activation of AGT is a necessary and critical step in the progression of lung fibrogenesis (45).

**Cathepsin D.** Cathepsin D (CatD) is an aspartyl protease which can be found in all living cells but not in the red blood cells (49). Proteolytic cleavages of pre-pro-enzyme CatD (52 kDa) forms a heavy (34 kDa) and light chain (14 kDa) in the lysosomes at acidic pH (49). It was demonstrated that cathepsin D contains Asn-linked oligosaccharide/lobe, which serves as recognition domains for binding of lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (49).

**Angiotensin I (ANGI).** ANGI is an intermediate product of the RAS system. It is produced from AGT by aspartyl proteases such as renin or cathepsin D.

**Angiotensin converting enzyme (ACE).** ACE is a type 1 transmembrane protein that converts decapeptide angiotensin I (ANGI) to generate octapeptide angiotensin II (ANGII) by removing dipeptides from the C-terminus of the peptide substrate.

**Angiotensin II (ANGII).** ANGII is believed to have profibrotic potential by its abilities to upregulate collagen expression in lung fibroblasts (50) and induction of AEC apoptosis (45). It is a key regulator of the angiotensin system by its biological functions through the two specific receptors: ANGII receptor type I ($AT_1R$) and ANGII receptor type II ($AT_2R$) (24). High concentration of ANGII has been found in normal rat lung (50). ANGII - a potent vasoconstrictor – increases blood pressure, stimulates vascular growth as well as hypertrophy in differentiated cells (78).

**Angiotensin type 1 and type 2 receptor ($AT_1R$ and $AT_2R$).** ANGII interacts with at least two receptors, $AT_1R$ and $AT_2R$. These cell surface receptors belong to the G protein-coupled receptor (GPCR) family. Many of the pathologic effects of ANGII are mediated through interaction with $AT_1R$, while its function via $AT_2R$ is less understood (100).

**Angiotensin converting enzyme-2 (ACE-2).** Human ACE-2 is type I transmembrane metalloprotease that shares 40% sequence homology to ACE. ACE-2 is more restricted in tissue distribution than ACE; it is found predominantly in the heart, kidneys and testes; but low levels are detected in various tissues (37). ACE-2 cleaves a single residue from ANGI to yield ANG1-9 or removes a single residue from ANGII to form ANG1-7 (24, 25). However, the affinity for ANGII is much stronger than for ANGI, thus the production of ANG1-7 is more favorable.

**Angiotensin 1-7 (ANG1-7).** ANG1-7 is a peptide product of the RAS which is formed by the conversion of ANGII by ACE-2. It reduces blood pressure, alters fluid absorption, causes vasodilation and reduces vascular growth *in vitro* and *in vivo* (78). ANG1-7 is the main counterpart to both the pressor and proliferative effects of ANGII.

**Mas.** Mas, a member of GPCR family, has been identified as a receptor for ANG1-7 and is produced by the *mas* oncogene.

Capillary blood vessels in the lung are a major site of high-level expression of ACE and ANGII production in the human body (36). Previous studies in experimental lung fibrosis from our laboratory have shown that bleomycin-induced apoptosis of AECs requires the autocrine synthesis of AGT, ANGII and both angiotensin receptors ($AT_1R$ and $AT_2R$, 43). A purified fragment of angiotensinogen (F1-F14) containing cathepsin D and ACE cleavage sites, applied to

AEC *in vitro* produced mature ANGII peptide and induced apoptosis (43). Treatment of rats with bleomycin drastically increased the level of ANGII. Consistent with this, co-administration of antisense oligonucleotides against AGT mRNA inhibited the synthesis of lung-derived AGT, thus prevented bleomycin-induced AEC apoptosis and lung fibrogenesis (83). Additionally, our laboratory demonstrated that AGT expression was upregulated in fibroblasts in response to TGF-β, through a mechanism requiring the involvement of the transcription factors hypoxia-inducible factor-1α (HIF1α) and Jun D (72).

In the local renin-angiotensin system of epithelial cells, cathepsin D is the primary aspartyl protease that converts AGT to ANGI. Previous work has suggested a role of cathepsin D in the apoptosis of a variety of cells treated with etoposide or adriamycin, including kidney cell lines, PC12 cells (pheochromocytoma cells), leukemia and U1752 lung cancer cells (43). Cathepsin D is synthesized as a pre-pro-enzyme in the endoplasmic reticulum. It gets glycosylated before being transported to the Golgi and ultimately to the endosomal/lysosomal compartment (49). Although, it was originally found as an enzyme involved in the clearance of proteins, recent studies have suggested its role in autophagy and apoptosis (49). Inhibition of cathepsin D with pepstatin A or knockdown of cathepsin D mRNA using antisense oligonucleotides prevented the apoptosis of AECs in response to these inducers by inhibiting caspase-3 activation and nuclear fragmentation (43).

Our previous studies have reported that octapeptide ANGII, which is synthesized from its precursor AGT through cleavage activities of cathepsin D and ACE, is a potent inducer of apoptosis in AECs. Uhal *et al.* have reported that the apoptotic response to bleomycin (44), Fas

ligand (97) or TNF-α (94) required the autocrine generation of ANGII, which is produced constitutively by AECs. Wang and colleagues suggested that ANGII acts as a proapoptotic factor in AECs via $AT_1$ and $AT_2$ receptors (96). Suppression of ANGII effect using ACE inhibitors (captopril, enalapril, lisinopril) or $AT_1$ receptor blockers (candesartan, losartan) reduced fibrogenesis induced with bleomycin, gamma irradiation or the antiarrhythmic agent, amiodarone. Moreover, $AT_2$ receptor blocker PD123319 also showed similar effects on radiation- and bleomycin-induced lung fibrosis in rats and mice (86). Additionally, studies from our laboratory have demonstrated that apoptosis of AECs in response to ANGII is mainly mediated through $AT_1$ receptor by protein kinase C (PKC), which activates JNK phosphorylation. We showed that ANGII-induced apoptosis of primary rat AECs can be prevented by PKC inhibitor chelerythrine (67).

ACE-2, a homologue of ACE, cleaves the Phenylalanine from decapeptide ANGI to give rise to octapeptide ANGII. ACE-2 therefore functions as a negative regulator of the angiotensin system by inactivating ANGII. ACE-2 has been demonstrated to play a critical role in the pathogenesis of lung disease. In a study of bleomycin-induced apoptosis of AECs from our laboratory, ACE-2 was shown as a protective factor by its abilities to a) degrade the pro-apoptotic peptide ANGII thus limiting its accumulation and b) generate the anti-apoptotic peptide ANG1-7, which inhibits AEC apoptosis in both human and experimental lung fibrosis (42). Silencing by knockdown or competitive inhibition of ACE-2 (DX600) increased the steady-state concentrations of ANGII and decreased ANG1-7 (86).

Many studies have reported that the imbalance in the levels of extracellular ANGII and ANG1-7 contributes to the pathogenesis of lung disease (16). Our laboratory has found that the baseline levels of ANG1-7 is more abundant than ANGII in cell culture media of mouse lung AECs under unstimulated conditions, which is suggested as a protective mechanism for cell survival (16). Pretreatment of AECs with ANG1-7 could inhibit JNK phosphorylation in response to ANGII or bleomycin (86). ANG1-7 also prevents AEC apoptosis in response to these inducers by inhibiting caspase activation and nuclear fragmentation of AECs. This blocking activity is suggested to be mediated through ANG1-7 receptor *mas*. Although some data suggest that ANG1-7 can act through $AT_1R$ as well (104), ligand binding data have proved that ANG1-7 has low affinity for the $AT_1$ or $AT_2$ receptors (14). It is demonstrated that A779, a specific blocker of the ANG1-7 receptor *mas*, could inhibit the anti-apoptotic effect of ANG1-7 (86) through an unknown mechanism. Additionally, our recently published study reported that ANG1-7 activates mitogen-activated protein kinase phosphatase (MKP). Blockade of MKP-2 significantly upregulates JNK phosphorylation, caspase activation and DNA fragmentation, thus preventing the apoptotic inhibition of ANG1-7 (16).

Numerous studies have confirmed the ability of ANG1-7 to inhibit ANGII activity and its proapoptotic effect. Experiments on primary cultures of rat proximal tubular cells have shown that ANG1-7 blocked ANGII-induced phosphorylation of p38, ERK1/2 and JNK, and partially inhibited ANGII-stimulated activation of TGF-β (86). Additionally, ANG1-7 reversed the stimulation of proximal tubule $Na^+$-ATPase activated by ANGII in perfused kidney preparations (86). ANG1-7 also blocks the ANGII-induced activation of MAP kinase and upregulates a tyrosine phosphatase (86). However, blockade of ANG1-7 by specific *mas* antagonist or by knockdown of

*mas* could prevent this inhibitory effect. Taken together, it is demonstrated that ANG1-7, the anti-apoptotic product of ACE-2, regulates the survival of AECs by blocking the activity of ANGII, inhibiting the JNK phosphorylation and AEC apoptosis through the ANG1-7 receptor *mas*.

**SURFACTANT PROTEIN C MUTATIONS**

There are four surfactant proteins that are divided into two groups: surfactant protein A (SP-A) and surfactant protein D (SP-D) are large and hydrophilic proteins, whereas surfactant protein B (SP-B) and surfactant protein C (SP-C) are small and hydrophobic proteins (4). SP-A and SP-D belongs to the collectin family that have carbohydrate-binding domain and plays an important role in innate lung host defense (57), while SP-B and SP-C plays a role in surface tension reduction. Alveolar stability of the lung is regulated by the phospholipid-rich layer (pulmonary surfactant) at the air-liquid interface which reduces surface tension to very low levels as alveolar surface area decreases (98).

Among the four surfactant proteins, SP-C is secreted exclusively by type II AECs (56). Human SP-C is synthesized as a 197-amino acid proprotein (proSP-C, 21 kDa) following alternative splicing of the primary transcript (98). SP-C then is processed into a 3.7 kDa mature form by a sequence of proteolytic cleavages (3, 102). Mature SP-C is secreted into the alveolar surface together with lipids and other surfactant components (102). SP-C is a type II integral membrane protein, with the C-terminal domain resides in the lumen of endoplasmic reticulum and the N-terminal domain resides in the cytosol (98). The cytosolic N-terminal domain of proSP-C is involved in the trafficking from the ER to secretory organelles, while the C-terminal

domain plays an important role in SP-C synthesis (54). Expression of proSP-C mutants lacking one or both conserved cysteine residues in the C-terminal-flanking region leads to the generation of misfolded SP-C proteins that will result in the formation of aggresomes (54).

Mutations in the SP-C result in the production of misfolded or unfolded proteins that are not processed via the secretory pathways. This leads to the formation of ubiquitins and aggresomes that can cause injury in many cells and tissues (30). Recent reports have suggested an association between mutations in the SP-C and pulmonary fibrosis. It is demonstrated that overexpression of SP-C C-terminal mutations in human (A549) and mouse (MLE12) lung epithelial cells caused accumulation of misfolded proteins, resulting in ER stress and apoptosis of AECs (38, 56). Additionally, it is reported that SP-C mutations are involved in the epithelial-mesenchymal transition (EMT) process in type II alveolar cells. Zhong *et al.* have shown that epithelial cells in response to thapsigargin, tunicamycin or overexpression of SP-C mutant underwent EMT process, which give rise to myofibroblasts, key effector cells in IPF, thus contributing to the fibrosis (103).

To date, there are more than 60 SP-C mutations identified in both children and adults with lung disease (57). These SP-C mutations can be divided into three groups: (1) BRICHOS C-terminal mutations, (2) non-BRICHOS C-terminal domain mutations, and (3) cytoplasmic N-terminal domain mutations (46). Among these, SP-C mutations in the BRICHOS domain (L188Q, G100S, Δexon4) have been well documented to play an important role in inflammation and apoptosis of AECs (46, 47, 56, 61, 80). Table 1.1 summarizes 10 SP-C mutations and their effects on AEC viability.

**Table 1.1. Fibrogenic SP-C BRICHOS domain mutations and AEC death.** [1]

| Mutation (in sequence order) | G100S | P115L | ΔExon4 | A116D | Q145H | T187N | L188P | L188Q | C189Y | L194P |
|---|---|---|---|---|---|---|---|---|---|---|
| **Causes ILD** | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| **Cause AEC Death** | yes | ? | yes | yes | ? | ? | ? | yes | ? | ? |
| **UPR markers** | increased | ? | increased | ? | ? | ? | ? | increased | ? | ? |
| **ER Stress**<br><br>**(Bip, HSPs)** | increased | reduced[82] | increased | reduced[82]<br><br>increased[102] | ? | ? | ? | increased[38]<br><br>reduced[82] | ? | ? |
| **References** | 61,88 | 60, 82,101 | 47,56 | 82,102 | 17 | 101 | 17 | 38,55,82 | 17,81 | 17 |

[1.] This data was published on the following manuscript: Uhal B., Nguyen H. The Witschi hypothesis revisited after 35 years: Genetic proof from SP-C BRICHOS domain mutations. *Am J Physiol Lung Cell Mol Physiol* 305(2):L906-11, 2013.

## ENDOPLASMIC RETICULUM STRESS AND UNFOLDED PROTEIN RESPONSE IN DISEASES

The ER is a membrane bound organelle responsible for the synthesis, translocation and folding of secreted and membrane proteins. When the protein folding mechanism is overwhelmed, ER stress occurs and activates the UPR. The UPR signaling pathway comprises of three ER transmembrane sensors (Fig. 1.3): activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1α), and protein kinase RNA-like ER kinase (PERK). Under basal conditions, these three proteins are bound to ER chaperone GRP78/BiP (immunoglobulin-heavy-chain binding protein) and remain inactive (41). Upon severe or prolong ER stress, BiP is released from these sensors thus activating the UPR (28).

**IRE1.** Mammalian IRE1 has two isoforms: IRE1α and IRE1β. Whereas IRE1α can be found in most cells and tissues, IRE1β is strictly expressed in intestinal epithelial cells (28). IRE1 is a dual-activity type I transmembrane protein which has a serine-threonine kinase domain and an endoribonuclease domain (77). Once activated, IRE1α dimerizes and autophosphorylates, which leads to the activation of the endonuclease domain. Activated IRE1α then removes a 26-nucleotide intron from the X-box-binding protein 1 (XBP1) mRNA, which results in the production spliced form of XBP-1 (sXBP1).

**PERK.** PERK is an ER transmembrane protein with a cytosolic (serine/threonine) kinase domain. In response to ER stress, PERK undergoes dimerization and autophosphorylation. This results in the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) on its Ser51 residue (48) which attenuates global protein synthesis, but also allows the selective translation of activating ATF4 (transcription factor 4) and its downstream targets such as CHOP

(transcriptional factor C/EBP homologous protein) and GADD34 (growth arrest and DNA damage-inducible 34) (73).

**ATF6.** ATF6 is a type II transmembrane protein which has a basic leucine zipper (bZIP) transcription factor in its cytosolic domain. Upon ER stress, ATF6 dissociates the GRP78 from the ER and translocates to the Golgi apparatus, where it is cleaved by the serine protease site-1 (S1P) and by metalloprotease site-2 protease (S2P) to release the cytosolic portion of ATF6 (22).

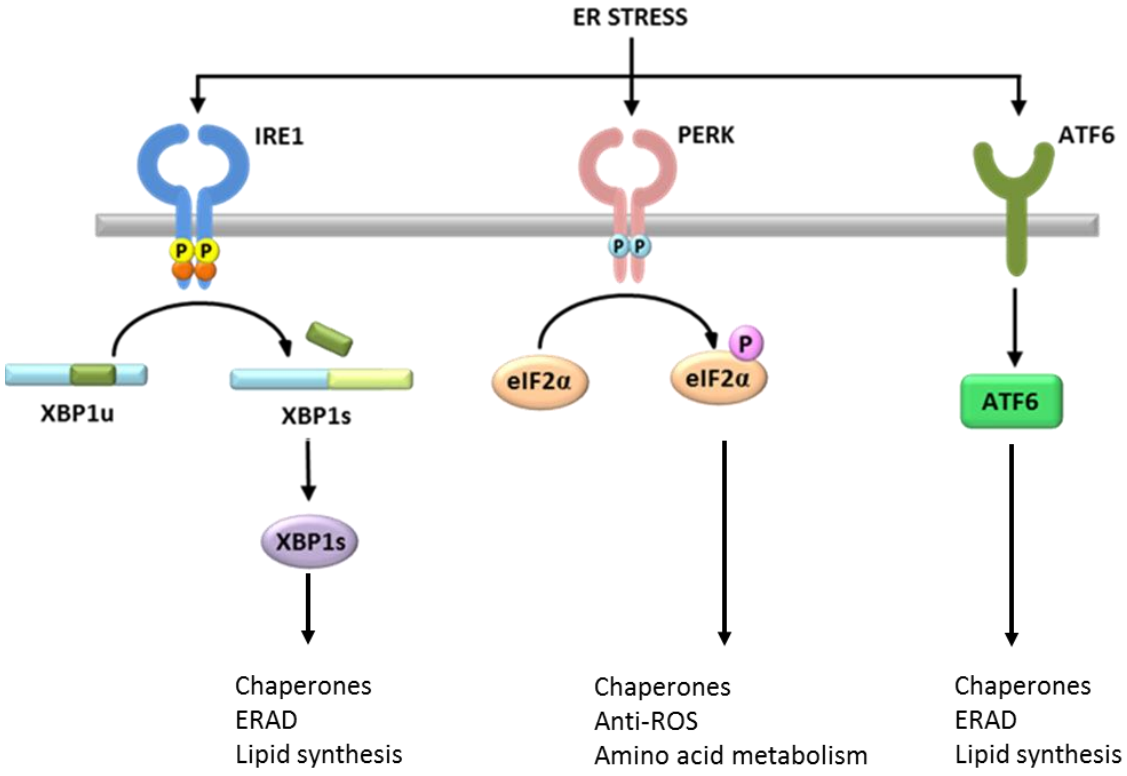


**Figure 1.3. ER stress and the unfolded protein response.** Under ER stress, BiP releases from three ER stress sensors, thus activates the three pathways of the UPR: activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and PKR-like ER kinase (PERK). Cleaved ATF6 upregulates the transcription of genes that increase protein-folding capacity, protein degradation and lipid synthesis. Activation of PERK blocks the global protein synthesis, but enables the translation of ATF4, thus induces the transcription of targets that are required for the ER homeostasis and amino acid synthesis. Splicing of XBP1 initiates the transcription of many chaperones as well as genes involved in protein degradation. ROS: Reactive oxygen species; ERAD: ER-associated protein degradation.

ER stress and UPR signaling have been well documented in numerous diseases including pulmonary fibrosis, diabetes, neurodegeneration, inflammatory disorders, cancer as well as heart diseases, suggesting that ER stress-induced apoptosis is an important event in pathophysiological conditions (48, 77).

***ER stress and metabolic diseases***

ER stress and UPR signaling have been shown to be involved in regulation of glucose and lipid metabolism. Nonalcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (ALD) are the two major liver diseases worldwide. Treatment of mice with tunicamycin caused lipid accumulation in hepatocytes, which activated all three sensors of UPR (71). It is suggested that UPR activation promotes NAFLD by stimulatimg lipogenesis, enhances lipolysis, decreases lipoprotein secretion and reduces fatty-acid oxidation (93). XBP1s modulates fatty acid synthesis by upregulating the expression of lipogenic enzymes, and downregulating hepatic gluconeogenesis via interaction with forkhead box protein O1 (FOXO1) and phosphoinositide 3-kinase (PI3K) (21).

ER stress and UPR were also shown to contribute to the pathogenesis of diabetes. Dysregulation of the PERK and IRE1α pathways has critical consequences for the survival of β-cells (48). It is demonstrated that Wolcott-Rallison syndrome, a rare human diabetic syndrome, is caused by the loss-in-function mutations in PERK that leads to β cell dysfunction (92). PERK deficient mice have also been proved to be more prone to diabetes and hyperglycemia (19). Recent studies reported that ER stress induces JNK-mediated phosphorylation of insulin receptor substrate 1 (IRS1), a substrate of insulin receptor, thus limit insulin activity (64).

***ER stress and lung diseases***

The activation of ER stress and UPR was first reported in patients with familial interstitial pneumonia (FIP) who carried mutations in SP-C (33). SP-C protein mutations (primarily in the C-terminal domain, were the first to be deciphered in familial forms of idiopathic interstitial pneumonia (IIP) (18). Observations of IPF human lungs suggested that SP-C mutations cause accumulation of misfolded proteins, leading to caspase activation and mitochondrial cytochrome *c* release, which ultimately results in AEC apoptosis (55, 56). Moreover, ER stress has been shown to play an important role in epithelial-to-mesenchymal transition, a source of fibroblasts and myofibroblasts in fibrotic lungs. Tanjore *et al.* suggested that ER stress activates Smad2/3 and Src-dependent signaling pathways, which could be prevented by siRNA targeting of IRE1α (79, 80).

About 90% of cystic fibrosis (CF) cases were caused by the ΔF508 mutation on the ion channel protein, cystic fibrosis transmembrane conductance regulator (CFTR). ΔF508 mutation produces incorrectly folded CFTR proteins which accumulate within the ER and are essentially targeted for degradation via the proteasome (99). It was demonstrated that human epithelial cells transfected with ΔF508-CFTR activated UPR markers such as GRP78/BiP and ATF6 in response to exogenous UPR inducers thapsigargin and tunicamycin (29).

***ER stress and cancer***

A variety of cancers have been documented to be associated with ER stress and UPR activation. Cancer cells usually arise, invade and metastasize in environments under stressful conditions. Recent studies demonstrated the involvement of PERK and IRE1 arms in tumor

growth - XBP1 and PERK deficient cells had reduced ability to form solid tumors (48). IRE1α regulates the expression of pro-inflammatory cytokines and angiogenic proteins, thus affecting the tumor growth and invasion in glioblastoma. ATF6, however, has been shown to play pivotal role in the survival and adaptation of squamous carcinoma cells to chemotherapy, nutritional stress and *in vivo* microenvironment (21). Components of ER protein-folding machinery have been reported to induce tumor progression, cell survival and metastasis of many cell types. Among those, GRP78/BiP expression may serve as a biomarker of cancer progression (40).

***ER stress and neurodegenerative disorders***

UPR has been reported to be associated with several neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Accumulation of misfolded proteins alters neuronal connectivity, leads to neuronal cell death and dysfunctional ER-associated protein degradation (ERAD) machinery (6). In the brains of Alzheimer's patients and mice models, it was observed that there was an accumulation of amyloid-β (Aβ) peptides, as well as the upregulation of phosphorylated eIF2α and ATF4 (93). Additionally, studies of brain tissue from mouse models and from patients affected with Pelizaeus-Merzbacher diseases (PMDs) have shown a correlation between the accumulation of protein aggregation and upregulation of ER stress markers (21). Abrogation of ATF6 increases the susceptibility of dopaminergic neurons to neurotoxins that induce Parkinson's disease (21, 70).

***ER stress and inflammatory disorders***

Unfolded or misfolded protein accumulation was observed in autoimmune diseases such as rheumatoid arthritis, inflammatory bowel diseases and multiple sclerosis (6). IRE1, XBP1

or PERK plays critical role in pathological progression of Crohn's disease and ulcerative colitis. XBP1 mRNA splicing was required for maximal production of pro-inflammatory cytokines, especially interleukin-6 in macrophages (21). UPR pathways also regulate cell differentiation and protein secretion in B cells and dendritic cells (51).

## INTERFERING WITH ER STRESS AND UPR

Given the strong evidence of ER stress and UPR in a variety of diseases, a number of efforts have been made to identify the pharmacological modulators that control cell fate under ER stress conditions. In many cases, it was suggested that switching between cell homeostatic survival and apoptosis by enhancing the adaptive capability of stressed cells or reducing protein misfolding in the ER could be a potential strategy. PERK and IRE1α with their enzyme active sites have drawn strong interest in finding small molecules which act as modulators for these proteins.

### *Chemical chaperones*

Chemical chaperones are small compounds that are used to improve ER folding capacity and stabilizing protein conformation (34). The most studied chemical chaperones are tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyrate (4-PBA), which have been reported to attenuate ER stress in various cell types and tissues. The Food and Drug Administration (FDA) has approved 4-PBA for treatment of biliary cirrhosis and TUDCA for treatment of urea cycle disorders. Abrogation of ER stress with 4-PBA was shown to be protective in macrophages

against lipotoxic death associated with atherosclerosis by inhibiting *XBP1* splicing and CHOP expression (12). TUDCA has been demonstrated to reduce ethanol-induced liver damage in human hepatoblastoma Hep G2 cells (58). These chaperone also improved insulin sensitivity and glucose homeostasis in *in vivo* mouse models and in diabetic patients (65). Moreover, Ozcan *et al.* have shown that 4-PBA and TUDCA pretreatment increase the leptin sensitivity of both genetic and diet-induced obesity models (63).

***PERK inhibitors***

Numerous efforts have been made to identify compounds that inhibit the kinase activity of PERK by optimizing ATP-competitive inhibitors. GlaxoSmithKline has recently developed novel PERK inhibitors that can prevent the autophosphorylation of PERK and phosphorylation of eIF2α in mouse and human cells in response to ER stress (35). Among those, GSK2606414, also known as compound 38, is an ATP-competitive inhibitor of PERK that also inhibits PERK phosphorylation, thus decreasing tumor growth in mouse models of pancreatic cancer (2). GSK2656157 is also a small molecule that inhibits ER stress-induced PERK autophosphorylation, eIF2α substrate phosphorylation, together with decreased in ATF4 and CHOP in human pancreatic adenocarcinoma (1).

***IRE1α inhibitors***

A number of efforts have been made to identify small molecules that can directly inhibit IRE1α. Pharmacological strategies have focused on the two active sites of this sensor: the catalytic core of the RNase domain and the ATP-binding pocket of the kinase domain.

The most effective ligands for targeting the RNase domain of IRE1α have been proven to be salicylaldehydes and its derivatives (4μ8C, MKC-3946 and STF-083010) (48). Among those, MKC-3946 inhibits XBP1 splicing resulting in the inhibition of tumor growth in mice models of multiple myeloma (53). Treatment with STF-08310 has also been shown to significantly inhibit growth of tumors in mice models of multiple myeloma. Cross *et al.* have identified 4μ8C as a small molecule that targets the critical lysine 907 residue in the catalytic core of the IRE1α RNase domain, forming a stable imine that blocks XBP1 mRNA splicing and IRE1-mediated mRNA degradation (7). These salicylaldehydes were believed to inhibit IRE1α endonuclease activity without affecting IRE1α dimerization and oligomerization. In addition to salicylaldehydes, recent studies have discovered a new small molecule inhibitor of IRE1α RNAse activity from the culture broth of an *Actinomycete* strain. This compound, toyocamycin, was shown to inhibit thapsigargin- and tunicamycin-XBP1 mRNA splicing in Hela cells without affecting IRE1α phosphorylation or ATF6 and PERK activity (69). Toyocamycin was also demonstrated to suppress growth of xenografts in *in vivo* model of human multiple myeloma (69).

Similar to PERK inhibitors, ATP-competitive blockers that target the kinase domain of IRE1α have also been identified. Type I ATP-competitive inhibitors (1NM-PP1, sunitinib, APY29) are small compounds that interact with the ATP-binding pocket, thus preventing IRE1α autophosphorylation, but were also shown to activate the RNase domain. Papa *et al.* have identified 1NM-PP1 as a ligand that blocks IRE1α kinase activity while being able to restore IRE1α's ability to cleave RNA (66). Similarly, sunitinib and APY29 were reported to be inhibitory to IRE1α phosphorylation but stimulatory to *XBP1* mRNA splicing (21).

Recent discoveries have identified KIRAs (Kinase-Inhibiting RNase Attenuators) as type II ATP-competitive ligands which inhibit IRE1α RNase activity by breaking oligomers. Among those, KIRA3 has been shown to suppress the dimerization-oligomerization of IRE1α by stabilizing the monomeric form of this protein. It was discovered that KIRA6 displayed greater efficacy in inhibiting IRE1α in vivo and promoting cell survival than KIRA3. This potent inhibitor preserves photoreceptor viability in rat models of ER stress-induced retinal degeneration (15). Treatment with KIRA6 also preserves pancreatic β cells by upregulating insulin and decreasing hyperglycemia in Akita diabetic mice (15). In contrast to type I ATP-competitive inhibitors, these type II IRE1α ligands inhibit both the kinase and RNase activities of IRE1α (48).

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## CHAPTER 2

## PREVENTION OF ER STRESS-INDUCED APOPTOSIS OF ALVEOLAR EPITHELIAL CELLS BY ANGIOTENSIN1-7[1]

[1] The work described in this chapter was published as the following manuscript: Bruce D. Uhal, Hang Nguyen, My-Trang Dang, Indiwari Gopallawa, Vinh Dang, Shinji Ono and Konosuke Morimoto. Abrogation of ER stress-induced apoptosis of alveolar epithelial cells by angiotensin 1-7. *Am J Physiol Lung Cell Mol Physiol* 305: L33-41, 2013.

**ABSTRACT**

Recent work reported that dysfunctional type II alveolar epithelial cells (AECs) play critical role in the pathogenesis of idiopathic pulmonary fibrosis (IPF). It is demonstrated that apoptosis of AECs in response to endogenous or xenobiotic agents is regulated by the autocrine angiotensin system. Previous studies have shown that mutations in the BRICHOS domain of surfactant protein C (SP-C) can cause the accumulation of misfolded proteins in the endoplasmic reticulum (ER), lead to ER stress and unfolded protein response (UPR) activation, thus promote the pathogenesis of lung diseases. In this study, we hypothesized that ER stress induced AEC apoptosis, which can be prevented by manipulation of the ANG system of AECs. Human A549 cells or primary cultures of human AECs were treated with proteasome inhibitor (MG132). MG132-induced ER stress increases ANGII-generating enzyme cathepsin D or decreases ANGII-degrading enzyme ACE-2. Apoptosis of AECs was measured by caspase activation and nuclear fragmentation. It was shown that ER stress-induced apoptosis was prevented by ANG receptor blocker saralasin or by antiapoptotic peptide ANG1-7. Also, apoptotic blocking activity of ANG1-7 was eliminated by the specific *mas* antagonist A779. The finding herein demonstrated that ER stress-induced apoptosis of AECs is mediated by the autocrine ANGII/ANG1-7 system. It also suggests that manipulation of the ANG system might lead to therapeutic strategies to limit alveolar epithelial cell death.

**INTRODUCTION**

A critical event in the initiation and progression of pulmonary fibrosis is the apoptosis of alveolar epithelial cells (AECs, 8, 12). This concept was first proposed over 30 years ago by Haschek and Witschi (3). Support for this theory demonstrated that inhibition of caspases (6, 26) or deletion of genes critical to apoptosis (1) could prevent experimental lung fibrogenesis. Moreover, injury of alveolar epithelial type II cells (AECs) with subsequent cell death has been reported to be sufficient for the progression of fibrosis in mice lungs (4, 18).

Recent studies from our laboratory reported that exposure of AECs to apoptotic inducers such as Fas ligand (27), TNF-α (25) or bleomycin (13) can upregulate angiotensinogen (AGT) mRNA and protein, the 58 kDa protein precursor of angiotensin II (ANGII). ANGII is produced enzymatically by cleavage of the decapeptide angiotensin I (ANGI) from AGT by renin or aspartyl protease (22). Blockade of apoptosis by ANG receptor blockers and antisense oligonucleotides against AGT demonstrated that autocrine synthesis of ANGII is required for the apoptosis of ACEs in response to the apoptotic agents (13, 25, 26). Apoptosis of AECs in response to ANGII is mediated by $AT_1$ receptor, which can be blocked by $AT_1$-selective blockers Losartan (21). Losartan was shown to significantly reduce caspase-3 activation in bleomycin-administrated to mice (21).

Angiotensin converting enzyme 2 (ACE-2), which degrades the profibrotic octapeptide ANGII to form heptapeptide ANG1-7, is protective for the survival of AECs by limiting the local accumulation of ANGII in response to bleomycin (22). Primary cultures of rat AECs showed that knockdown of ACE-2 by small interfering RNA (siRNA) or by competitive inhibition with peptide

DX600 can exacerbate the apoptosis of AECs in response to bleomycin (21). Previous studies demonstrated that the counterregulatory axis composed of ACE-2, ANG1-7 and its receptor *mas* controls apoptosis of AECs by regulating the autocrine ANGII generation (24). It was reported that ANG1-7 inhibits ANGII- or bleomycin-induced apoptosis of AECs by preventing the JNK phosphorylation (2).

A variety of lung irritants such as cigarette smoke, environmental pollutants, infectious microorganisms can cause the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (28). Many studies have demonstrated that ER stress can lead to apoptosis of AECs and subsequent lung fibrosis. It is recently discovered that mutations in the BRICHOS domain of surfactant protein C (SP-C), which is exclusively secreted by alveolar epithelial type II cells, can cause ER stress and activate the unfolded protein response (UPR) - eventually leading to AEC cell death (5, 7, 15).

This study tested the hypothesis that ER stress-induced apoptosis of AECs might be regulated by the autocrine ANGII/ANG1-7 system. The findings herein reported that ER stress altered the autocrine ANGII/ANG1-7 system causing AEC apoptosis, which could be controlled by using the ANG receptor blocker saralasin or antiapoptotic peptide ANG1-7.

## MATERIALS AND METHODS

### *Reagents and materials*

Proteasome inhibitor MG132, ANG receptor nonselective blocker saralasin and propidium iodide were purchased from Sigma Chemical (St Louis, MO). Angiotensin 1-7 and A779 (D-Ala$^7$-Ang1-7) were obtained from GenScript USA (Piscataway, NJ). Cathepsin D fluorogenic substrate was purchased from Peptides International (Louisville, KY). Antibodies for Western blotting of GRP78/BiP, CHOP, active forms of caspase-7 and caspase-8, cytochrome *c* and β-actin were obtained from Cell Signaling (Danvers, MA). ACE-2 antibodies were obtained from Abcam (Eugene, OR). Cathepsin D, anti-rabbit-HRP, anti-goat, anti-mouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other materials were obtained from Sigma Chemical or were of reagent grade.

### *Cell culture*

Human lung adenocarcinoma cell line A549 was obtained from ATCC (Manassas, VA) and were grown in 6- or 24-well chambers in Ham's F-12 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum, 1% total volume of penicillin and streptomycin. When the desired confluency was reached through inspection under microscopy, cells were serum-starved for 24 hours before exposed to MG132 for 20 hours.

### *Western blotting*

A549 cells were collected in ice-cold Nonidet P-40 –based lysis buffer containing protease inhibitor cocktail (Complete Mini, Roche, Nutley, NJ). Whole-cell lysates were

centrifuged at 11,000g for 15 minutes at 4$^{o}$C and supernatants were collected for protein detection. Proteins were loaded into polyacrylamide gels, ran at 120 V and then transferred to PVDF membrane. Membranes were washed with TBS containing 0.1% Tween (TBST) before being blocked with 5% non-fat dry milk for 1 hour at room temperature. Membranes were then incubated with primary antibodies at 4$^{o}$C overnight and washed three times with TBST buffer before incubated with secondary antibodies. Immunoreactive bands were detected using SuperSignal West Pico Cheluminescent substrate (ThermoScientific, Rockford, IL). Bands were quantified with densitometry using ImageJ (NIH). β-actin was used as loading control.

For detection of cytochrome *c*, A549 cell extracts were obtained in ice-cold cytosol extract lysis buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 mM HEPES). The lysis buffer was adjusted to pH 7.6 then added the protease inhibitor. Cell suspensions were centrifuged at 800g for 10 minutes at 4$^{o}$C to remove cell debris. Cell supernatants which contain cytosolic extract were transferred to a fresh tube and centrifuged at 11000g for 15 minutes at 4$^{o}$C to remove (something) and analyzed for cytosolic cytochrome *c* release.

***Assay of Cathepsin D activity***

The cathepsin D fluorogenic substrate MOCaC-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Ard-$NH_2$ was used for enzymatic activity following the manufacturer's instructions. A549 cell lysates were collected from cell culture and were incubated in opaque 96-well plates in 1.0 M sodium acetate buffer (pH 4.0) containing 50 µM fluorogenic substrate of cathepsin D. The total volume of reaction solution is 100 µl (24). Initial rates of fluorescent product

formation were obtained from the slope of continuous readings which were linear with both time and protein concentration (24).

A549 cell lysates were prepared in 1.0 M sodium acetate buffer (pH 4.0) containing 50 µM fluorogenic substrate and incubated in opaque 96-well culture plates suitable for top reading on a plate reader. Initial rates of fluorescent product formation were obtained from the slope of continuous readings which were linear with both time and protein concentration, taken over 30 minutes following the addition of substrate (24).

***Detection of nuclear fragmentation***

After treatment with inhibitor, A549 cells were detected for apoptosis by nuclear fragmentation assay using propidium iodide (PI) as described earlier (11, 24). Human AECs were fixed with 70% ethanol following a DNase-free RNase digestion in phosphate buffer saline (0.5% v/v) containing 5 µg/ml of PI. 24-well culture vessels were centrifuged to retain the detached cells during fixation with ethanol (11). Cells were incubated at 37$^{o}$C for at least 20 min to digest RNA. PI binds to DNA by intercalating between the bases with little or no sequence preference and it is only permeant to dead cells. Apoptotic cells were scored as cells with discrete nuclear fragments containing condensed chromatin, in a minimum of four different microscopic fields from at least three culture vessels per treatment group; in situ end labeling (ISEL) of fragmented DNA was also examined to detect apoptotic fragmented nuclei of AECs in the nuclear fragment assay (11). Results were similar to the PI detection method. Caspase-7 and caspase-8 activation, and other markers of the apoptosis stage were also measured by Western blot using antibodies specific for the active (cleaved) form of caspase-7 and caspase-8 (24).

**RESULTS**

***Activation of ER stress markers upon MG132 induction***

Human A549 cells in response to proteasome inhibitor MG132 activated the ER stress markers by upregulating GRP78/BiP and CHOP proteins. Figure 2.1 showed the increase in the protein level of GRP78/BiP (panel A) and CHOP (panel B) in A549 cells treated with MG132 compared to the vehicle.

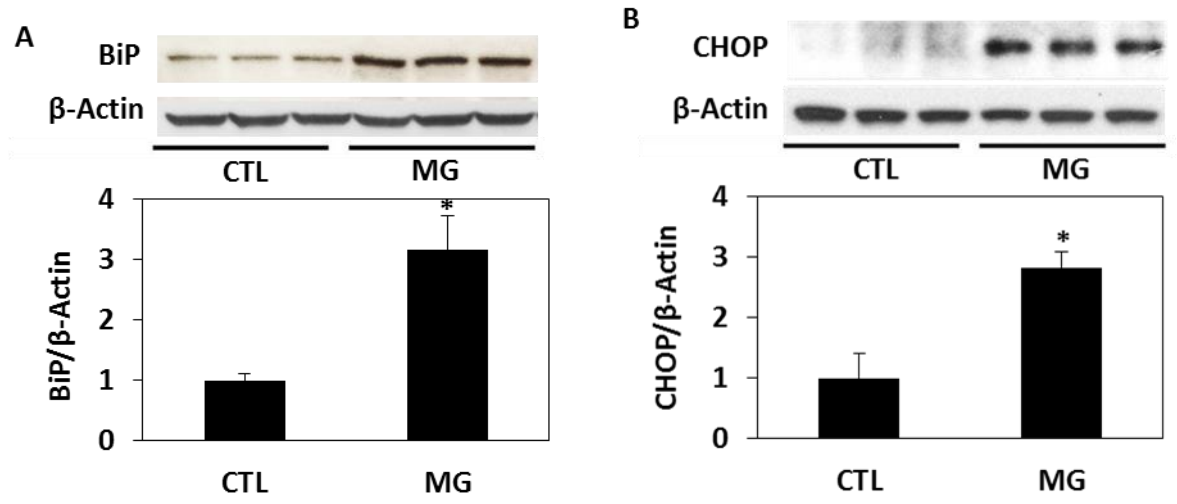


**Figure 2.1. Activation of ER stress markers in response to ER stress.** Human A549 cells were incubated with MG132 (10 μM) for 20 h. Whole-cell lysates were collected and analyzed for BiP (panel A) and CHOP (panel B) using Western blotting. β-actin was used as loading control. Bars represent the mean ± S.E.M. of at least six independent experiments; *=P < 0.05 vs. CTL by ANOVA and Student *t*-test.

***ER stress induced cathepsin D activation***

Previous studies from our laboratory have demonstrated that cathepsin D, an aspartyl protease that enzymatically cleaves AGT to generate ANGII, is required for the apoptosis of

AECs (24). Therefore, using the same concentration of MG132 that induced BiP and CHOP activation in Figure 2.1, the effects of ER stress on the alteration of cathepsin D in AECs was examined. Figure 2.2 shows that MG132-induced ER stress upregulates both the inactive and active forms of cathepsin D (panel A). Moreover, enzymatic activity using the specific cathepsin D fluorogenic substrate indicated that cathepsin D activity of A549 cells was significantly increased by MG132 and completely blocked by the addition of cathepsin D inhibitor pepstatin A (panel B, 24).

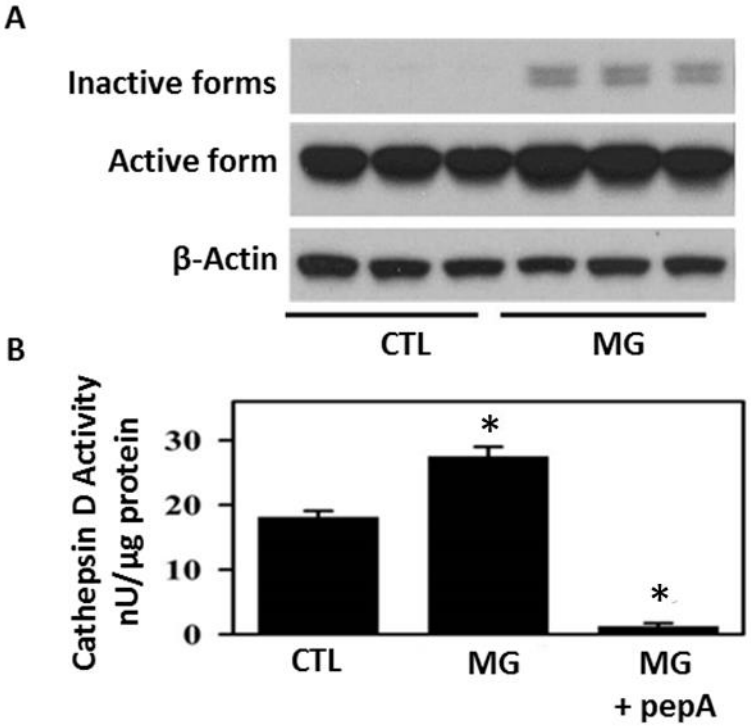


**Figure 2.2. Activation of cathepsin D by ER stress inducer in human AECs.** In panel A: Human alveolar epithelial cell line A549 was incubated with MG132 (10μM) for 20 h then whole-cell lysates were immunoblotted with cathepsin D antibodies; β-actin was used as loading control. In panel B: A549 cells were challenged with MG132 then whole-cell lysates were analyzed for cathepsin D enzyme assay in the presence or absence of pepstatin A (1μM). Bars represent the mean ± S.E.M. of at least six independent experiments; *=P < 0.05 vs. CTL by ANOVA and Student-Newman Keul's multiple comparison test (24).

***ER stress reduced ACE-2 activation***

Previous studies in our laboratory have demonstrated the role of the ACE-2/ANG1-7 systems in the regulation of bleomycin-induced apoptosis of AECs (22). We showed that ACE-2 is protective and required for the survival of AECs by converting the pro-apoptotic octapeptide ANGII to the anti-apoptotic heptapeptide ANG1-7 (9). Thus, the effect of MG132 on ACE-2 in human AECs were examined. After incubation with increasing concentrations of MG132 for 20 hours, A549 cells exhibited a dose-dependent down-regulation of ACE-2, beginning at a concentration of 7.5 µM (Figure 2.3, 24).

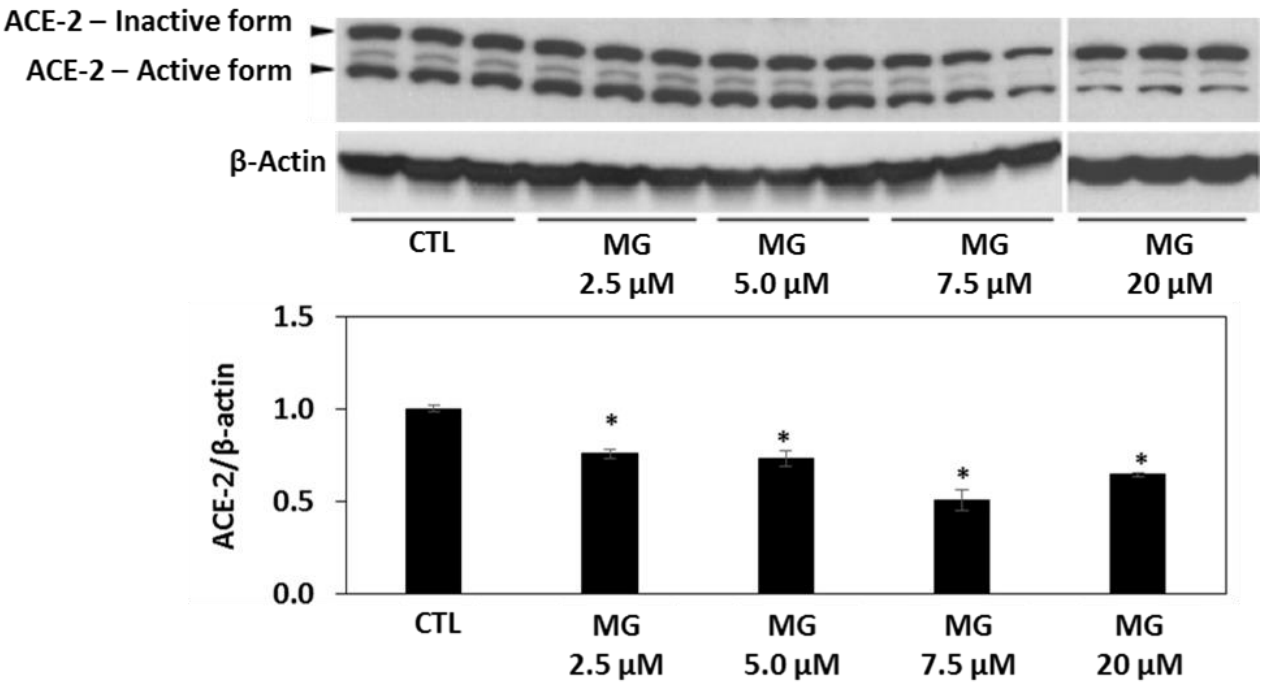


**Figure 2.3. Downregulation of ACE-2 by ER stress in human AECs.** Human alveolar epithelial A549 cell line was treated with MG132 at the indicated concentrations for 20 h. Whole-cell lysates were harvested for Western blotting using anti-ACE-2 antibodies. β-actin was used as loading control. Bars represent the mean ± S.E.M. of at least three independent experiments; *=P < 0.05 vs. CTL by ANOVA and Student-Newman Keul's multiple comparison test.

***Prevention of ER stress-induced caspase activation of AECs by ANG1-7 or by angiotensin nonselective receptor blocker saralasin***

Although the signaling mechanisms by which ER stress activates the angiotensin system to induce apoptosis were not defined yet, it seems likely that both axes of the angiotensin system are involved. While ANGII is a potent inducer of AEC apoptosis, its product by ACE-2, ANG1-7 is required for the survival of AECs (22, 27). In order to determine which arm of the angiotensin system is involved in the ER stress-induced apoptosis in human AECs, detection of caspase activation was performed in the presence or absence of ANG1-7 or angiotensin nonselective receptor blocker saralasin. Figure 2.4 showed that both ANG1-7 and saralasin significantly block the ER stress-induced caspase-7 activation of AECs (panel A). Moreover, it also demonstrated that ANG1-7 completely eliminated caspase-8 activation by MG132, while saralasin partly blocked the cleavage of caspase-8 (panel B).

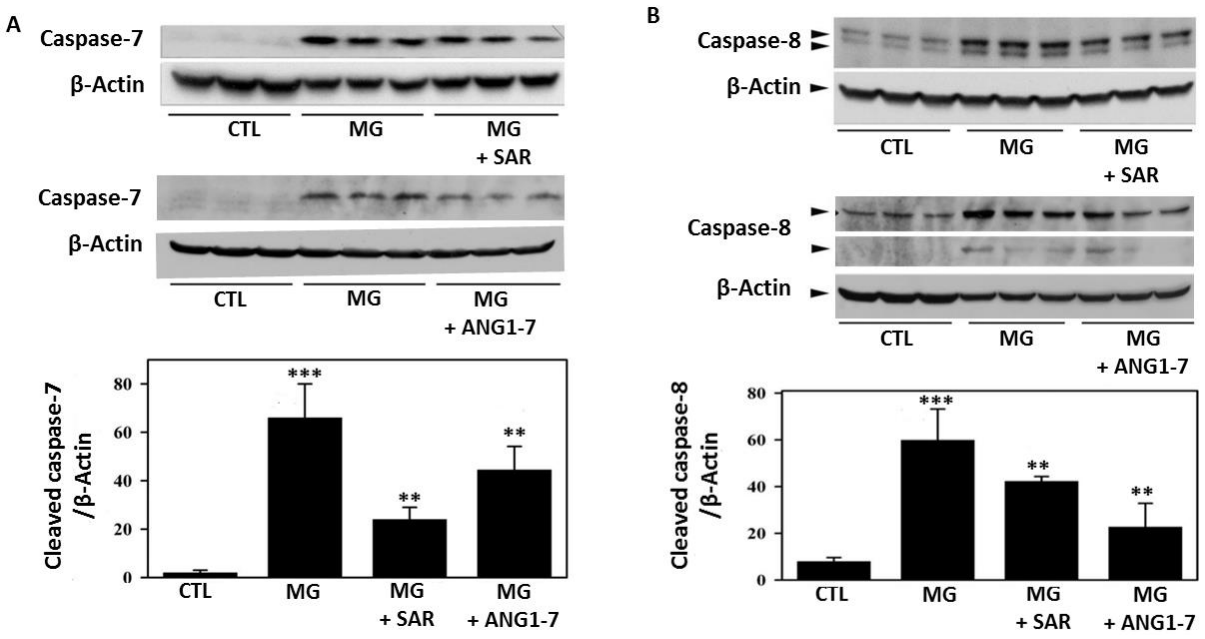


**Figure 2.4. Inhibition of MG132-induced caspase activation of human AECs by ANG1-7 or by angiotensin receptor blocker saralasin.** A549 cells were challenged with MG132 (10 μM) in the presence or absence of anti-apoptotic peptide angiotensin 1-7 (ANG1-7, $10^{-7}$M) or angiotensin nonselective receptor blocker saralasin (SAR, 50 μg/ml) for 20 h, each treatment was added 30 minutes before MG132 (24). Whole-cell lysates were harvested for detection of caspase activation using cleaved caspase-7 and caspase-8 antibodies. β-actin was used as loading control. Bars represent the mean ± S.E.M. of at least 3 separate cell cultures. For panel A: ***=P < 0.001 vs. CTL and **=P < 0.05 vs. CTL and vs. MG. For panel B: ***=P < 0.001 vs. CTL and **=P < 0.01 vs. MG but not significant vs. CTL (24). Significant differences are determined by ANOVA and Student-Newman Keul's multiple comparison test.

***Prevention of MG132-induced cytosolic cytochrome c by ANG1-7 or by angiotensin receptor blocker saralasin***

Mulugeta *et al.* demonstrated that ER stress induced the cytosolic release of cytochrome *c* during human AEC apoptosis (15, 16). Therefore, we tested whether anti-apoptotic ANG1-7 or ANGII nonselective blocker saralasin would block cytosolic cytochrome *c* release of AECs in response to MG132 treatment. A549 cells were challenged with MG132 in

the presence or absence of ANG1-7 or nonselective ANGII receptor blocker saralasin. Figure 2.5 showed that both saralasin or ANG1-7 strongly inhibited the release of cytosolic cytochrome *c* induced by MG132 (24). Thus protecting the human alveolar cells from apoptosis.

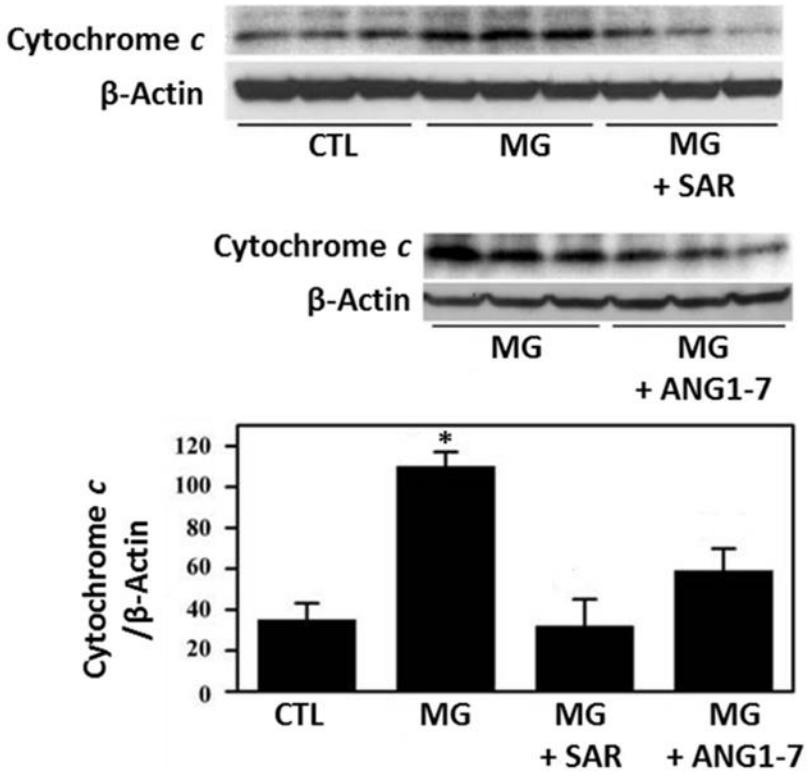


**Figure 2.5. Inhibition of MG132-induced cytochrome *c* release by ANG1-7 or by saralasin**. Human A549 cells were challenged with MG132 (10 μM) in the presence or absence of anti-apoptotic peptide angiotensin 1-7 (ANG1-7, $10^{-7}$M) or angiotensin nonselective receptor blocker saralasin (SAR, 50 μg/ml) for 20 h. Cytosolic fraction of the cell lysates were collected and detected for cytochrome *c* release by Western blotting. Bars are means ± SE of at least three cell cultures. *=P < 0.05 vs CTL using ANOVA and Student-Newman-Keuls post hoc test.

***Prevention of ER stress-induced nulear fragmentation of AECs by angiotensin nonselective receptor blocker saralasin or ANG1-7***

To examine the inhibitory activity of saralasin and ANG1-7 in AECs, A549 cells and primary cultures of human AECs were treated with MG132 and examined by a nuclear fragmentation assay to test the final stages of apoptosis (22). Saralasin strongly inhibited and ANG1-7 completely blocked MG132-induced nuclear fragmentation in either A549 cells or primary cultures of AECs (Figure 2.6, panel A) (24). Moreover, I also observed that ANG1-7 receptor *mas* antagonist A779 inhibited the blockade of nuclear fragmentation in primary cultures of human AECs (panel B).

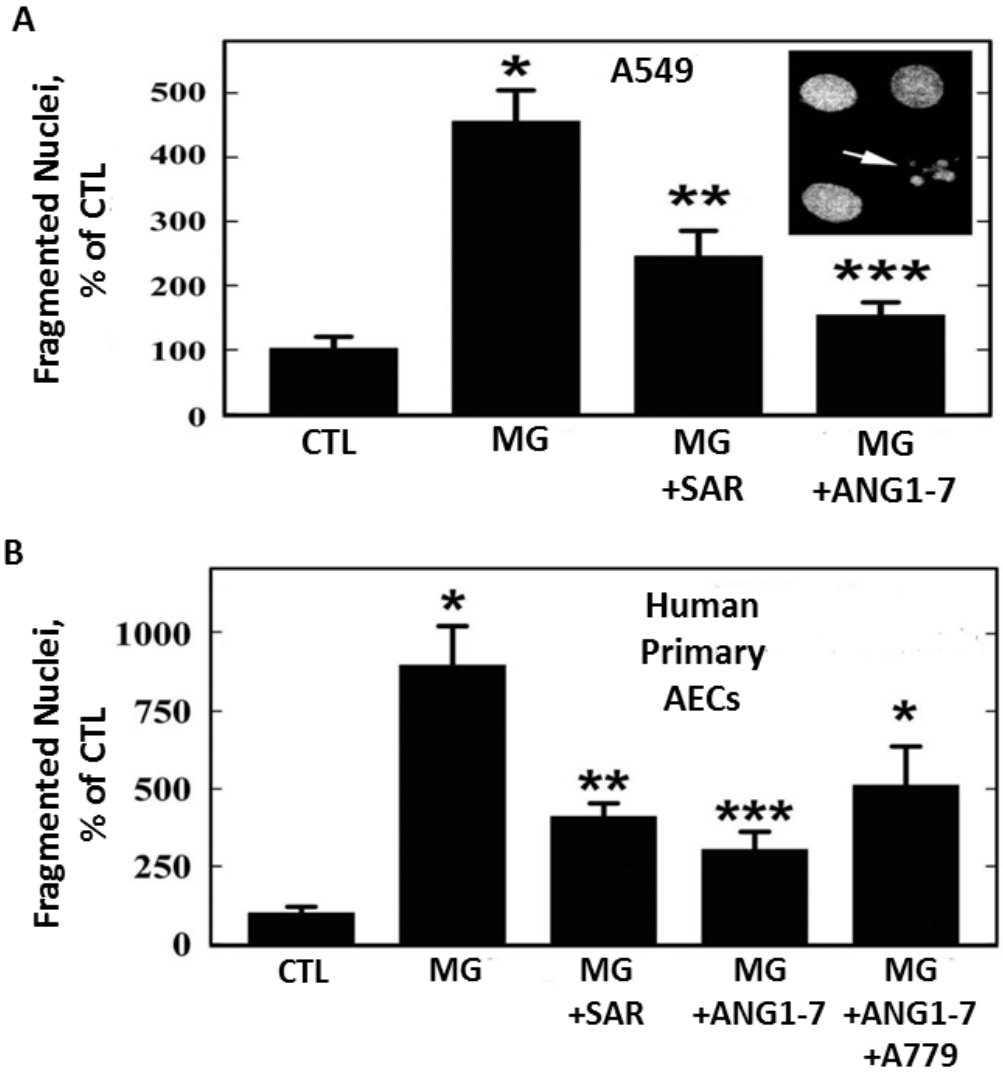


**Figure 2.6. Prevention of ER stress-induced nuclear fragmentation of AECs by ANG1-7 or by saralasin.** In panel A, A549 cells were challenged with MG132 (10 μM) in the presence or absence of anti-apoptotic peptide angiotensin 1-7 (ANG1-7, $10^{-7}$ M) or angiotensin nonselective receptor blocker saralasin (SAR, 50 μg/ml) for 20 h. In panel B, primary cultures of human alveolar epithelial cells were challenged with MG132 (10 μM) in the presence or absence of angiotensin nonselective receptor blocker saralasin (SAR, 50 μg/ml), or anti-apoptotic peptide angiotensin 1-7 (ANG1-7, $10^{-7}$M) with or without the specific mas antagonist A779 ($10^{-7}$M) for 20 h. Apoptotic cells were examined by microscopic quantitation of nuclear fragmentation with propidium iodide. Bars represent means ± SE of n = 6 in at least 4 cell cultures. *=P < 0.05 vs. CTL, ** =P < 0.05 vs MG and vs. CTL, ***= not significant vs. CTL but P < 0.05 vs. MG by ANOVA and and Student-Newman-Keuls post hoc test.

**DISCUSSION**

Recent reports from our laboratory demonstrated that the ACE-2/ANG1-7/*mas* axis regulates AEC apoptosis in response to pro-fibrotic apoptosis inducers ANGII or bleomycin (22). ACE-2 was demonstrated to have a protective effect through its abilities to decrease levels of the pro-apoptotic ANGII and increase levels of its anti-apoptotic product ANG1-7. We also showed that the inhibition of ACE-2 by small interfering RNA (siRNA) or by a competitive inhibitor (DX600 peptide) in AECs led to an increase in ANGII and a decrease in ANG1-7 in cell culture media thus inducing apoptosis (22). Additionally, our earlier report demonstrated that an increase in cathepsin D induced by bleomycin results in ANGII generation that led to apoptosis of AECs without an increase in angiotensinogen transcription (10). Therefore, our data indicates that an increase in cathepsin D or a decrease in ACE-2 induced by MG132 might be sufficient to explain the ER stress-induced apoptosis in AECs (24). However, the signaling mechanisms of how MG132 increases cathepsin D and decreases ACE-2 are still undefined.

Another way of inducing ER stress was transfecting A549 cells with mutant G100S SP-C. Our laboratory demonstrated that the mutant G100S BRICHOS domain SP-C showed an increase in ER stress marker BiP compared to the wild type SP-C (24), which is similar to what we obtained with cells treated with MG132. In agreement with MG132 treatment, we also observed that A549 cells transfected with the G100S mutant had a decrease in the active form of ACE-2 relative to wild type SP-C (24). It was also shown that the mutant G100S SP-C-induced nuclear fragmentation in AECs was eliminated by either saralasin or ANG1-7, and this protective effect of ANG1-7 was prevented by specific *mas* antagonist A779 (24), which is similar to the

results that we obtained in MG132-induced nuclear fragmentation study. These results suggest that the angiotensin system regulates AEC apoptosis in response to ER stress induced by either synthetic proteasome inhibitor (MG132) or by BRICHOS domain mutation of SP-C (G100S) (24). Thus, utilization of either MG132 or SP-C BRICHOS domain mutant will extend our understanding of the ER stress-induced apoptosis of human AECs.

The results reported herein demonstrated that MG132-induced apoptosis could be completely abrogated by anti-apoptotic peptide ANG1-7 or strongly inhibited by ANGII receptor blockade saralasin either in A549 cells or in primary cultures of human AECs. Moreover, either ANG1-7 or saralasin could also strongly block the nuclear fragmentation of A549 cells induced by G100S BRICHOS domain mutation of SP-C (24). The ability of ANG1-7 to inhibit nuclear fragmentation more potently than saralasin suggests that the ACE-2/ANG1-7/*mas* axis plays a more potent role in ER stress-induced apoptosis, relative to the ANGII/ANG receptor axis reported earlier (24). However, the finding that both protein level and enzymatic activity of cathepsin D, an ANGII-producing enzyme, were upregulated by MG132 suggests the possibility that ANGII receptor blockers might also play important role in the inhibition of ER stress-induced apoptosis. Therefore, understanding the mechanisms by which saralasin and ANG1-7 block apoptosis induced by ER stress, and defining which arm of the angiotensin system is most important to these processes are interesting topics for future investigations in our laboratory.

Numerous studies have reported the correlation between ER stress and UPR in many cell lines, but not specifically the activation of UPR induced by MG132 in A549 cells. Maguire *et al.* demonstrated that there was a significant increase in the activity of ATF6 in A549 cells with

plasmids containing sequences for Δexon4 BRICHOS domain mutation of SP-C versus the wild type human SP-C (14). Ono *et al.* also reported that mutant G100S-transfected A549 cells showed a significant increase in IRE1 and phospho-PERK immunoreactive protein (17). In support of this, Blackwell's group recently reported that the IRE1 arm of the UPR appears to be involved in activating the ER stress pathways in rat type II AEC, RLE6TN (19). These data strongly suggest the involvement of UPR in the apoptotic pathways of human AECs induced by proteasome inhibitor MG132.

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## CHAPTER 3

# THE UNFOLDED PROTEIN RESPONSE CONTROLS ER STRESS-INDUCED APOPTOSIS OF LUNG EPITHELIAL CELLS THROUGH ANGIOTENSIN GENERATION[1]

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**ABSTRACT**

Recent work from this laboratory showed that endoplasmic reticulum (ER) stress-induced apoptosis of alveolar epithelial cells (AECs) is regulated by the autocrine angiotensin (ANG)II/ANG1-7 system. The proteasome inhibitor MG132 or surfactant protein C (SP-C) BRICHOS domain mutation G100S induced apoptosis in human AECs by activating the pro-apoptotic cathepsin D and reducing anti-apoptotic angiotensin converting enzyme-2 (ACE-2). This study tested the hypothesis that ER stress-induced apoptosis of human AECs might be mediated by influence of the unfolded protein response (UPR) on the autocrine ANGII/ANG1-7 system. A549 cells were challenged with MG132 or SP-C BRICHOS domain mutant G100S to induce ER stress and activation of UPR pathways. The results showed that either MG132 or G100S SP-C mutation activated all 3 canonical pathways of the UPR (IRE1/XBP1, ATF6, and PERK/eIF2α), which led to a significantly increase in cathepsin D or in TACE – an ACE-2 ectodomain shedding enzyme - and eventually caused AEC apoptosis. However, ER stress-induced AEC apoptosis could be prevented by chemical chaperone or by UPR blockers. It is also suggested that ATF6 and IRE1 pathways might play important role in regulation of angiotensin system. These data demonstrate that ER stress induces apoptosis in human AECs through mediation of UPR pathways, which in turn regulate the autocrine ANGII/ANG1-7 system. They also demonstrated that ER stress-induced AEC apoptosis can be blocked by inhibition of UPR signaling pathways.

## INTRODUCTION

Apoptosis of alveolar epithelial cells (AECs) contributes to the pathogenesis of acute lung injury, pulmonary fibrosis and chronic obstructive pulmonary disease (15). The concept that apoptosis is critical to lung disease pathogenesis is supported by numerous studies showing, for example, that caspase inhibitors (9, 35) or deletion of genes critical to apoptosis (1) could prevent experimental lung injury and fibrogenesis. Recent findings indicate that BRICHOS domain mutations in surfactant protein C (SP-C) induce ER stress and apoptosis in type II AECs and lead to subsequent lung fibrogenesis (32). Therefore, understanding the pathogenesis of lung injury and fibrosis requires understanding of the regulation of AEC apoptosis.

Endogenous and xenobiotic inducers of AEC apoptosis lead to the autocrine conversion of angiotensin II (ANGII) from its precursor angiotensinogen (AGT, 16,34,36). The apoptotic response to Fas ligand, TNF-α or bleomycin requires the synthesis of ANGII, which is produced by AECs. Anti-sense oligonucleotides against AGT mRNA or neutralizing antibodies against ANGII were sufficient to block AEC apoptosis (16, 34, 36). Moreover, studies of lung biopsy specimens from patients with pulmonary fibrosis showed a dramatic increase in AGT mRNA and protein, which suggests that the generation of ANGII is important for human lung fibrosis (12).

The octapeptide ANGII is produced by the sequential cleavage of the decapeptide angiotensin I (ANGI) from the N-terminal end of AGT by cathepsin D, followed by a subsequent cleavage of two amino acids from C-terminal end of ANGI. This peptide is then further processed by removal of one final C-terminal amino acid by angiotensin converting enzyme 2

(ACE-2) to yield the heptapeptide angiotensin1-7 (ANG1-7, 13). In concert with ANGII production, recent work in our laboratory showed that the ACE-2/ANG1-7/mas axis also plays an important role in regulating of AEC apoptosis (31). In a study of bleomycin-induced apoptosis of AECs, ACE-2 was shown as a protective factor by its abilities to a) degrade the pro-apoptotic peptide ANGII thus limiting its accumulation and b) generate the anti-apoptotic peptide ANG1-7, which inhibits AEC apoptosis through the ANG1-7 receptor mas (31).

Surfactant protein C (SP-C) is the cleaved product of a precursor protein (proSP-C), which is synthesized by alveolar epithelial type II cells. Recent studies showed that the proSP-C contains a domain known as BRICHOS, which is thought to have chaperone-like properties that protect the peptide from aggregation. Mutations in the BRICHOS domain result in a product that cannot be processed normally in type II AECs, leading to accumulation of misfolded proSP-C in the endoplasmic reticulum (ER). SP-C BRICHOS mutants (such as SP-$C^{G100S}$, SP-$C^{L188Q}$ and SP-$C^{\Delta exon4}$) have been shown to induce the ER stress and activate the unfolded protein response (UPR), which lead to cell death and subsequent lung fibrosis (17, 22, 24).

The UPR is a signal transduction pathway that protects eukaryote cells from stress caused by the accumulation of unfolded or misfolded proteins in the ER (28). However, in the event of prolonged or severe ER stress that is not resolved, the UPR switches from protection of the cell to initiation of apoptosis (7). The UPR is comprised of signaling cascades that are governed by three ER transmembrane proteins: inositol-requiring element 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6), which remain inactive under non-stress conditions through the association with glucose regulated protein

78/immunoglobulin heavy-chain-binding protein (GRP78/BiP, 27). On accumulation of misfolded or unfolded proteins, BiP dissociates from these three sensors, which leads to their activation and triggers the UPR (26).

This study examined the hypothesis that ER stress-induced apoptosis of human AECs might be mediated by UPR pathways that in turn modulate the autocrine ANGII/ANG1-7 system of these cells. The results reported herein demonstrate that ER stress induced by either chemical agent (MG132) or SP-C BRICHOS domain mutation (G100S) leads to the activations of all three UPR pathways which result in an increase in cathepsin D and simultaneous activation of ACE-2 ectodomain shedding enzyme ADAM17/TACE, all of which can be prevented by the chemical chaperone sodium 4-phenylbutyrate (4-PBA).

## MATERIALS AND METHODS

### *Reagents and materials*

Synthetic proteasome inhibitor MG132 (carboxybenzoxy-Leu-Leu-leucinal), chemical chaperone sodium phenylbutyrate (4-PBA), IRE1 chemical inhibitor 4μ8C (8-formyl-7-hydroxy-4-methylcoumarin) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). PERK chemical inhibitor GSK2656157 was purchased from Santa Cruz (Dallas, TX). Antibodies for Western blotting were obtained as following: total IRE1, total PERK, total eIF2α, cleaved caspase-3, ATF4, ADAM17/TACE, CHOP, β-actin (Cell Signaling, Beverly, MA), ACE-2, phospho-eIF2α, phospho-IRE1 (Abcam, Cambridge, MA), XBP1, phospho-PERK, ATF6, cathepsin

D (Santa Cruz, Dallas, TX). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or BioRad Co. (Melville, NY).

***Cell culture***

The A549 human lung adenocarcinoma cell line was purchased from ATCC (Manassas, VA) and were grown in 6- or 24-well chambers in Ham's F-12 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Denville, Holliston, MA), 100 units/ml penicillin, 100 μg/ml streptomycin. The human primary alveolar epithelial cells were studied at day 2 of culture when they are morphologically and biochemically like type II cells (33). All cells were grown in 6-well or 24-well chambers and analyzed with inhibitors when they reached 70-80% confluency. For 4-PBA studies, cells were either incubated with 4-PBA (5 mM) for 1 h before treated with MG132 (10 μM) for 24 h or transfected with SP-C plasmids for 8 h prior to treatment with 4-PBA for 48 h. For specific UPR inhibition, A549 cells when reached 70-80% confluent were exposed to 4μ8C (50 μM) or GSK2656157 (1 μM) for 1 h before treated with MG132 (10 μM) for 24 h; or transfected with SP-C plasmids for 48 h before challenged to 4μ8C or GSK2656157 for 24 h.

***G100S mutant and wild-type SP-C plasmid transfection***

Human wild-type and G100S mutant SP-C DNA sequences carried in pIRES-dsRED plasmid were a kind gift from K. Morimoto of the Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University (Nagasaki, Japan). Wild-type and G100S-containing plasmids were amplified using Plasmid Plus Maxi Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. A549 cells grown to 70-80% confluence were transiently

transfected with the indicated plasmid construct using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY) as previously described (33).

***Western blotting***

A549 cells were collected in ice-cold protein lysis buffer (50 nM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate and protease inhibitor cocktail (Complete Mini, Roche, Nutley, NJ)). Cell suspensions were centrifuged at 11000g for 15 min at 4$^{o}$C and supernatants were collected for protein detection. Protein samples were run on 7.5–12% SDS gradient polyacrylamide gels and transferred to PVDF membranes. Membranes were incubated in 5% nonfat dry milk buffer in 1 h at RT, incubated with primary antibodies at 4$^{o}$C overnight then with secondary antibodies for 1 h at RT. Detection of the proteins was performed using Supersignal West Pico chemiluminescent substrate (GE Healthcare Biosciences, Pittsburgh, PA). β-actin antibodies were used as loading control.

***Knockdown of UPR pathway using siRNAs***

Antisense oligonucleotides against each human UPR sensors (ATF6, IRE1, PERK) were purchased from Santa Cruz (Dallas, TX). A549 cells were grown in 6-well chambers until reached 60-70% confluency then were transfected with antisense oligonucleotides against each target sensor (siATF6, siIRE1, siPERK) or with scrambled oligonucleotides (siCTL) by using lipofectamine 2000 (Invitrogen, Grand Island, NY). The oligonucleotide-to-lipofectamine ratio was optimized by using FITC-labeled nucleotides (31) and transfection efficiencies were determined by Western blotting (data not shown). Human A549 cells were transfected with

antisense oligonucleotides against UPR sensors for 24 h then treated with MG132 for 24 h or transfected with SP-C mutation for 48 h.

***Detection of apoptosis***

After treatment with inhibitor, A549 cells were monitored for apoptosis by nuclear fragmentation assay using propidium iodide (PI) as described earlier (16, 33). Human AECs were fixed with 70% ethanol following a DNase-free RNase digestion in phosphate buffer saline containing 5 μg/ml PI. 24-well culture vessels were centrifuged to retain the detached cells during fixation with ethanol (16). Apoptotic cells were scored as cells with discrete nuclear fragments containing condensed chromatin, in a minimum of four different microscopic fields from at least three culture vessels per treatment group; in situ end labeling (ISEL) of fragmented DNA was examined for equating of apoptotic fragmented nuclei of AECs in the nuclear fragment assay (16). Caspase-3 activation, another marker of the apoptosis stage was also measured by Western blot using antibodies specific for the active (cleaved) form of caspase-3 (31).

**RESULTS**

Recent work from our laboratory has shown that either the synthetic proteasome inhibitor MG132 or the G100S mutation of SP-C could upregulate GRP78/BiP (32), which interacts with all three components of the UPR signaling pathways under cellular homeostatic conditions. Previous studies also demonstrated that sodium 4-phenylbutyrate (4-PBA), a well-known chemical chaperone, could rescue the mutant SP-C protein from trafficking and reduce

ER stress in multiple cell types (25, 37). Therefore, we tested the role of 4-PBA in the regulation of UPR activation of AECs in response to proteasome inhibitor MG132 or to SP-C BRICHOS domain mutation G100S under similar conditions as previously reported (33).

***MG132- or G100S SP-C mutation-induced ER stress activates IRE1 pathway***

In response to ER stress, GRP78 dissociates from IRE1 which then undergoes dimerization and autophosphorylation. The data in Figure 3.1 showed a significant increase of phospho-IRE1 protein in A549 cells treated with MG132 (Fig. 3.1A), or in A549 cells transfected with G100S SP-C mutant compared to the wild-type SP-C-expressing cells (Fig. 3.1B). Upon activation, phosphorylated IRE1 protein induces the splicing of X-box binding protein 1 (XBP1) mRNA (11). A549 cells exposed to MG132 or transfected with the mutant G100S showed an elevation in the protein level of spliced XBP1, but this splicing was prevented by the effect of chemical chaperone 4-PBA (Fig. 3.1, C and D). These results suggest that blockade of the UPR pathways by 4-PBA could inhibit the activation of ER stress-induced IRE1/XBP1 pathways.

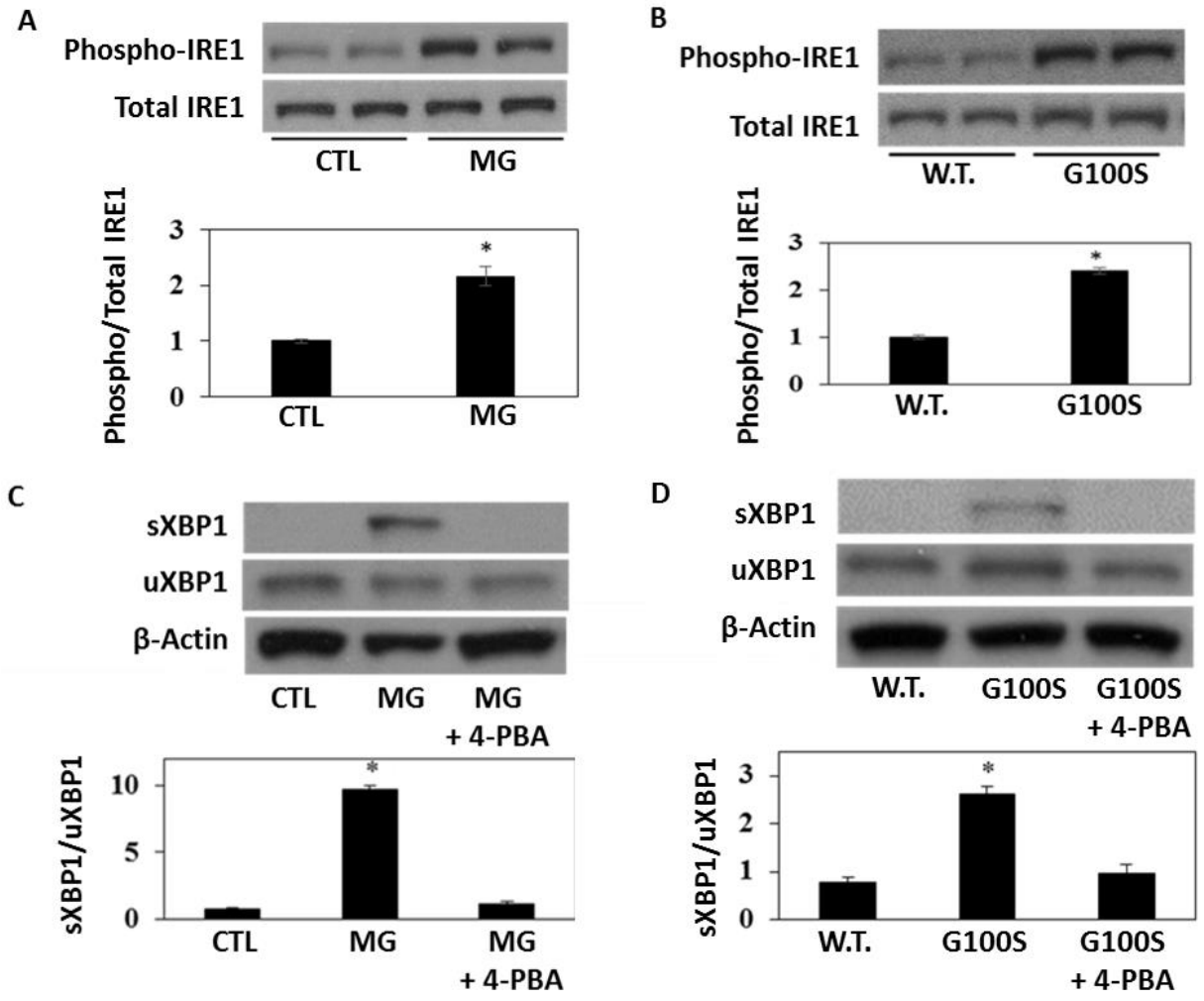


**Figure 3.1. Blocking of UPR pathways in AECs by 4-PBA can inhibit ER stress-induced activation of the IRE1 pathway.** Human alveolar A549 cells were either incubated with MG132 (10 μM, A and B) for 24 h or were transfected with wild-type SP-C (W.T.) or G100S SP-C mutation plasmids (C and D) for 48 h in the presence or absence of 4-PBA (5 mM). Whole-cell lysates were analyzed for phosphorylation of IRE1 (A and B) or splicing of XBP1 (C and D) using Western blotting. Bars represent mean ± S.E.M of at least three separate experiments. Significant differences were determined by ANOVA and Student-Newman Keul's test; *=P < 0.05 vs. CTL (A, C) or vs. W.T. (B and D).

***MG132- or G100S SP-C mutation-induced ER stress activates PERK pathway***

In order to identify the activity of the PERK pathway in response to ER stress, human alveolar A549 cells were challenged with synthetic ER stress inducer MG132 at the same concentration that activates the IRE1 pathway. As shown in Fig. 3.2A, the proteasome inhibitor MG132 strongly elevated the phosphorylation of PERK. This result is consistent with previous study by Ono and colleagues (22) who observed that A549 cells stably expressing the G100S mutation of SP-C, in the presence of proteasome inhibitor MG132, showed an increase in the level of phospho-PERK compared to the wild-type-expressing cells. Moreover and in agreement with the results shown above, the chemical chaperone 4-PBA significantly inhibited PERK phosphorylation in response to MG132 (Fig. 3.2A). Upon phosphorylation, the activated PERK phosphorylates the α-subunit of eukaryotic translational initiation factor 2 (eIF2α). Here we demonstrate that either MG132 (Fig. 3.2B) or SP-C BRICHOS domain mutation (Fig. 3.2C) substantially upregulated the phosphorylation of eIF2α protein, which was strongly inhibited by protein chaperone 4-PBA.

In response to ER stress, phosphorylated eIF2α induces the expression of Activating Transcription Factor-4 (ATF4) which in turn will induce the expression of another transcription factor, CCAAT/enhancer-binding protein homologous protein (CHOP, 27). However, in agreement with the results of Maguire and colleagues (17) who studied the Δexon-4 SP-C mutation, our previous work (33) showed that A549 cells transiently transfected with G100S SP-C mutation showed no significant increase in CHOP (33) or ATF4 protein (Fig. 3.2D) compared to wild-type SP-C. Also, although recent study from our laboratory have found that MG132

increased the level of CHOP protein in A549 cells (33), it is suggested that chemical chaperone 4-PBA could not block the activation of either ATF4 or CHOP protein in A549 cells (Fig. 3.2E).

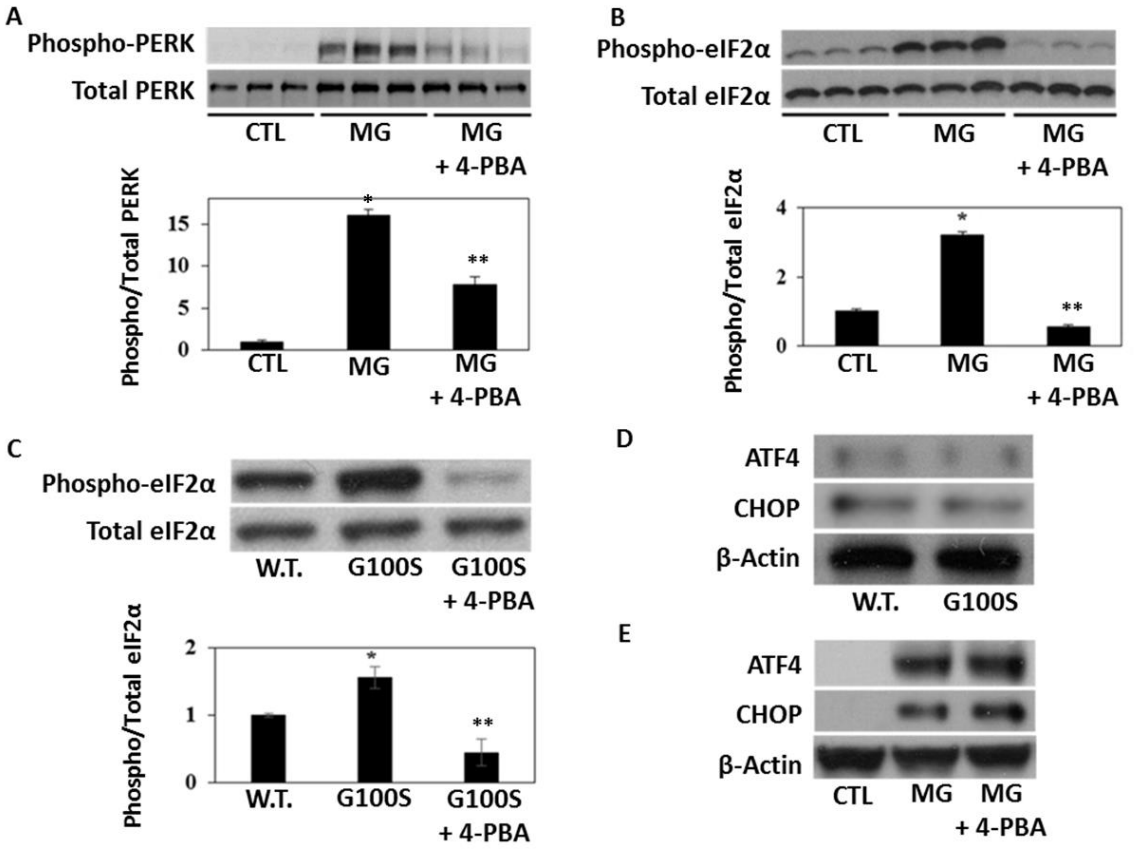


**Figure 3.2. Inhibition of the UPR pathways by chemical chaperone can prevent the PERK/eIF2α but not ATF4/CHOP activation.** Human A549 cells were incubated with MG132 (24 h for panel A, B; 12 h for panel E) or were transfected with plasmids containing wild-type SP-C or SP-C BRICHOS domain mutant G100S (48 h for panel C, 12 h for panel D) with or without chemical chaperone 4-PBA (5 mM). Whole-cell lysates were then harvested for detection of phospho-PERK and total PERK (A), phospho-eIF2α and total eIF2α (B, C), ATF4 and CHOP (D, E) using Western blotting. Anti β-actin antibody was used as loading control. Bars are the means + S.E.M of at least three separate experiments. *=P < 0.05 vs. CTL (A, B) or vs. WT (C); **= P < 0.05 vs MG (A, B) or vs. G100S by ANOVA and Student-Newman-Keul's multiple comparison test.

***MG132- or G100S SP-C mutation-induced ER stress activates ATF6 pathway***

In similarity to IRE1 and PERK, ATF6 binds to GRP78 and remains inactive under homeostatic conditions (11). In response to prolonged or severe ER stress however, ATF6 dissociates from BiP and is cleaved during the translocational process to generate the active form (11). Therefore, we next challenged A549 cells with ER stress inducers and assessed the level of ATF6 protein in response to ER stress in the presence or absence of 4-PBA. Figure 3.3 shows an increase of ATF6 protein in MG132-exposed A549 cells compared to vehicle-treated cells (Fig. 3.3A) or in mutant G100S-expressing cells compared to wild-type SP-C-expressing cells (Fig. 3.3B). However, this activation of ATF6 was prevented in the presence of sodium phenylbutyrate (Fig. 3.3).

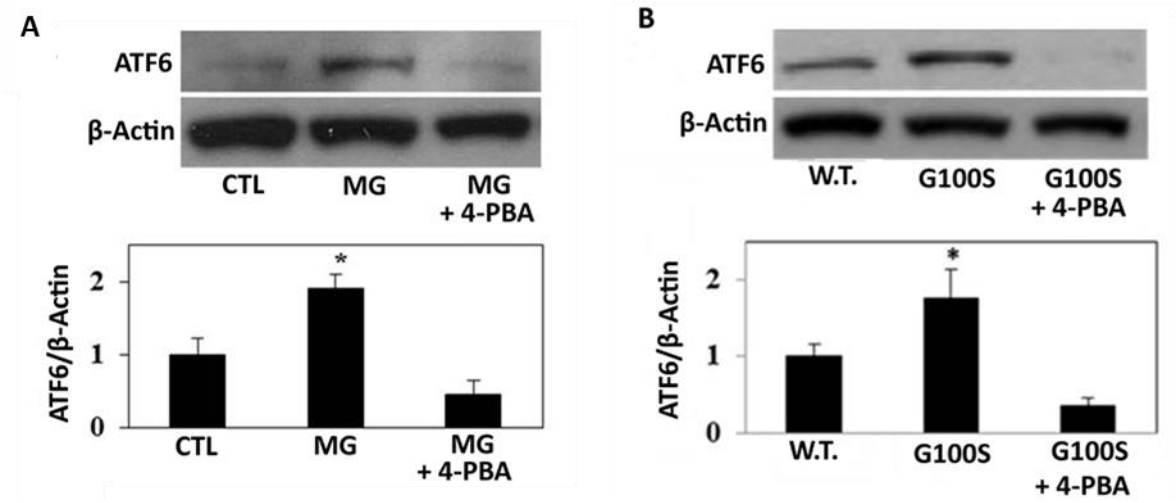


**Figure 3.3. Prevention of UPR pathways by 4-PBA can inhibit ER stress-induced ATF6 activation.** A549 cells were treated with proteasome inhibitor MG132 for 24 h (panel A), or were transiently transfected with plasmids containing wild-type SP-C or SP-C BRICHOS domain mutation G100S for 48 h (panel B) in the presence or absence of 4-PBA. Cells lysates were harvested for immunoblotting of ATF6 protein and β-actin was used as the loading control. Bars are the mean ± S.E.M of three independent experiments. *=$P < 0.05$ vs. CTL (A) or vs. W.T. (B) by ANOVA and Student-Newman-Keul's multiple comparison test.

***Chemical chaperone 4-PBA inhibits ER stress-induced cathepsin D activation***

Earlier work from our laboratory has shown that cathepsin D, an aspartyl protease that enzymatically cleaves angiotensinogen to produce ANGII, is required for AEC apoptosis (14, 33). Recent studies from this laboratory have demonstrated that either fibrogenic agent bleomycin (13) or ER stress inducer MG132 (33) upregulated both the immunoreactive protein and enzymatic activity of cathepsin D. Therefore, the ability of UPR to regulate cathepsin D was tested by challenging AECs with ER stress inducers with or without 4-PBA. Figure 3.4 shows that MG132-induced cathepsin D activation was significantly reduced by 4-PBA in either A549 cell line (Fig. 3.4A) or in primary cultures of human lung alveolar epithelial cells (Fig. 3.4B). In agreement with that result, A549 cells transfected with G100S SP-C mutation shows similar activation of cathepsin D by the G100S mutant, but reduction of cathepsin D (active form) upon exposure to the UPR chemical chaperone 4-PBA (Fig. 3.4C).

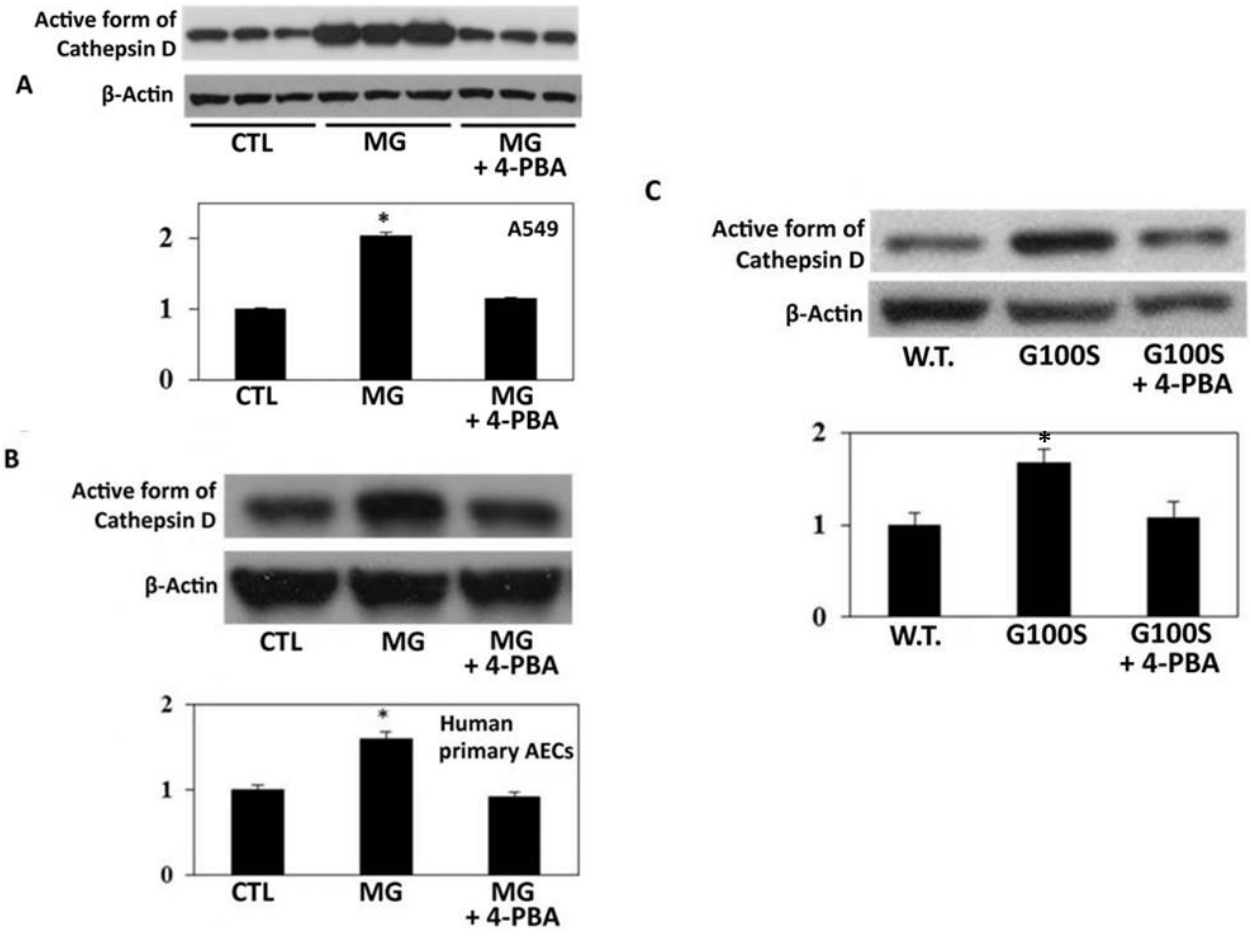


**Figure 3.4. Blocking of UPR pathways in AECs can prevent ER stress-induced cathepsin D activation.** In panel A and B, A549 cells (A) or primary culture of human alveolar epithelial cells (B) were treated with ER stress inducer MG132 (10 μM) for 24 h with or without 4-PBA (5mM); In panel C, A549 cells were transfected with plasmids containing wild-type (W.T.) SP-C or SP-C BRICHOS domain mutant (G100S) for 48 h in the presence or absence of 4-PBA (5mM). Whole-cell lysates were harvested for detection of cathepsin D protein, active form (14). Anti β-actin antibody was used as loading control. Results are the mean ± S.E.M of at least three cell cultures. *=P < 0.05 vs. CTL (A, B) or vs. W.T. (C) by ANOVA and Student-Newman-Keul's multiple comparison test.

***Chemical chaperone 4-PBA inhibits ER stress-induced ADAM17/TACE activation***

Our previous study proved that either MG132 or SP-C mutation decreased the activation of ACE-2 (33), thus we examined the level of ACE-2 in A549 cells challenged with ER stress inducers (MG132, G100S SP-C mutation) in the presence or absence of 4-PBA. Herein, we observed that ER stress-induced ACE-2 downregulation was potentially, but not significantly, prevented by chemical chaperone 4-PBA. On the other hand, studies from other groups have reported that ADAM17/TACE, a TNF-α-converting enzyme, can cleave and release the ectodomain of ACE-2 in several tissues, including the lungs (5, 21). Recent studies from our laboratory demonstrated that ADAM17/TACE is involved in the modulation of the angiotensin system (21, 33). Figure 3.5 showed that ER stress in response to MG132 (panel A) or G100S SP-C BRICHOS domain mutation (panel B) elevated the level of ADAM17/TACE in A549 cells; and this activation can be prevented by the chemical chaperone 4-PBA.

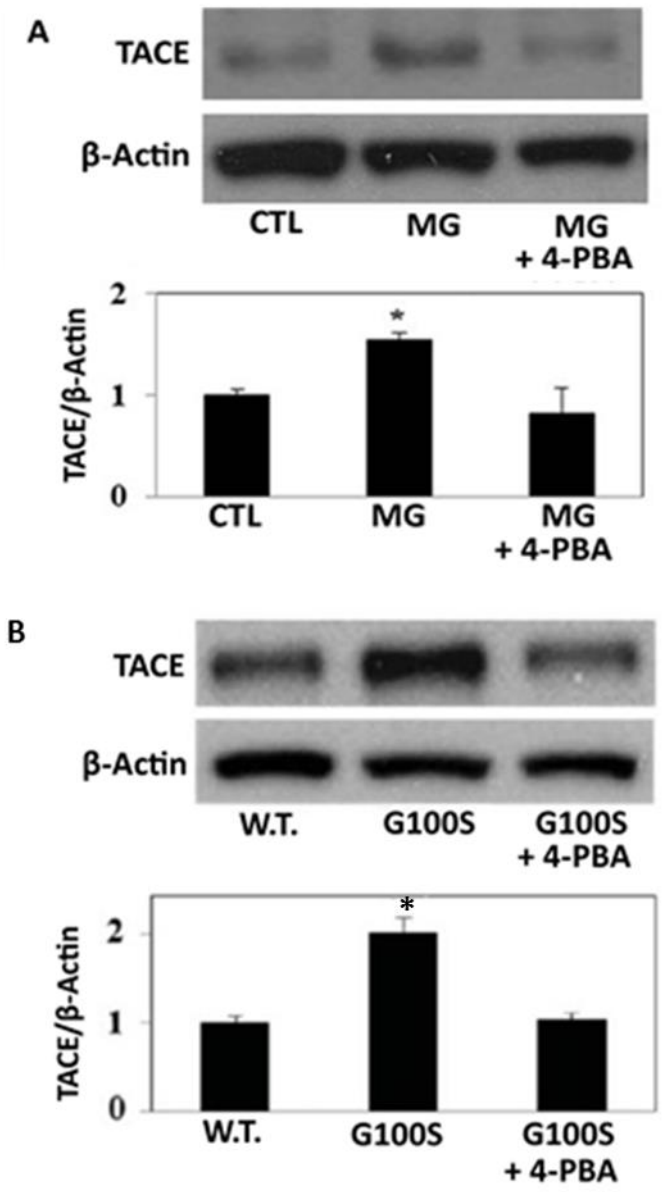


**Figure 3.5. Inhibition of the UPR pathways by chemical chaperone can inhibit the activation of ADAM17/TACE protein in AECs.** Human A549 cells were exposed to MG132 (10 μM) for 24 h (panel A) or were transfected with plasmids containing wild-type SP-C or SP-C BRICHOS domain mutant G100S for 48 h (panel B) with or without sodium 4-phenylbutyrate (5 mM). Whole-cell lysates were then harvested for detection of ADAM17/TACE and β-actin antibodies using Western blotting. Quantitation represents the mean ± S.E.M of at least three independent experiments. *=$P < 0.05$ vs. CTL (A) or vs. W.T. (B) by ANOVA and Student-Newman-Keul's post hoc test.

***Chemical chaperone 4-PBA inhibits ER stress-induced apoptosis of AECs***

*To test the role of 4-PBA in its effect on ER stress-induced apoptosis of AECs, A549 cells* were exposed to ER stress inducers, with or without 4-PBA, and were then assessed for caspase-3 activation. Figure 3.6 shows that either MG132 (Fig. 3.6A) or G100S SP-C mutation (Fig. 3.6B) strongly activated the cleavage of caspase-3, and this activation was significantly inhibited by chaperone 4-PBA. More importantly, human alveolar epithelial A549 cells were measured for nuclear fragmentation, a marker of the final stage of apoptosis. As shown in Figure 3.6, both MG132 and the G100S mutant significantly increased the nuclear fragmentation of A549 cells or human primary AECs, in agreement with our earlier results (33). More importantly, the UPR inhibitor 4-PBA significantly inhibited nuclear fragmentation induced by either MG132 (Fig. 3.6, C and D) or the SP-C BRICHOS domain mutation G100S (Fig. 3.6E).

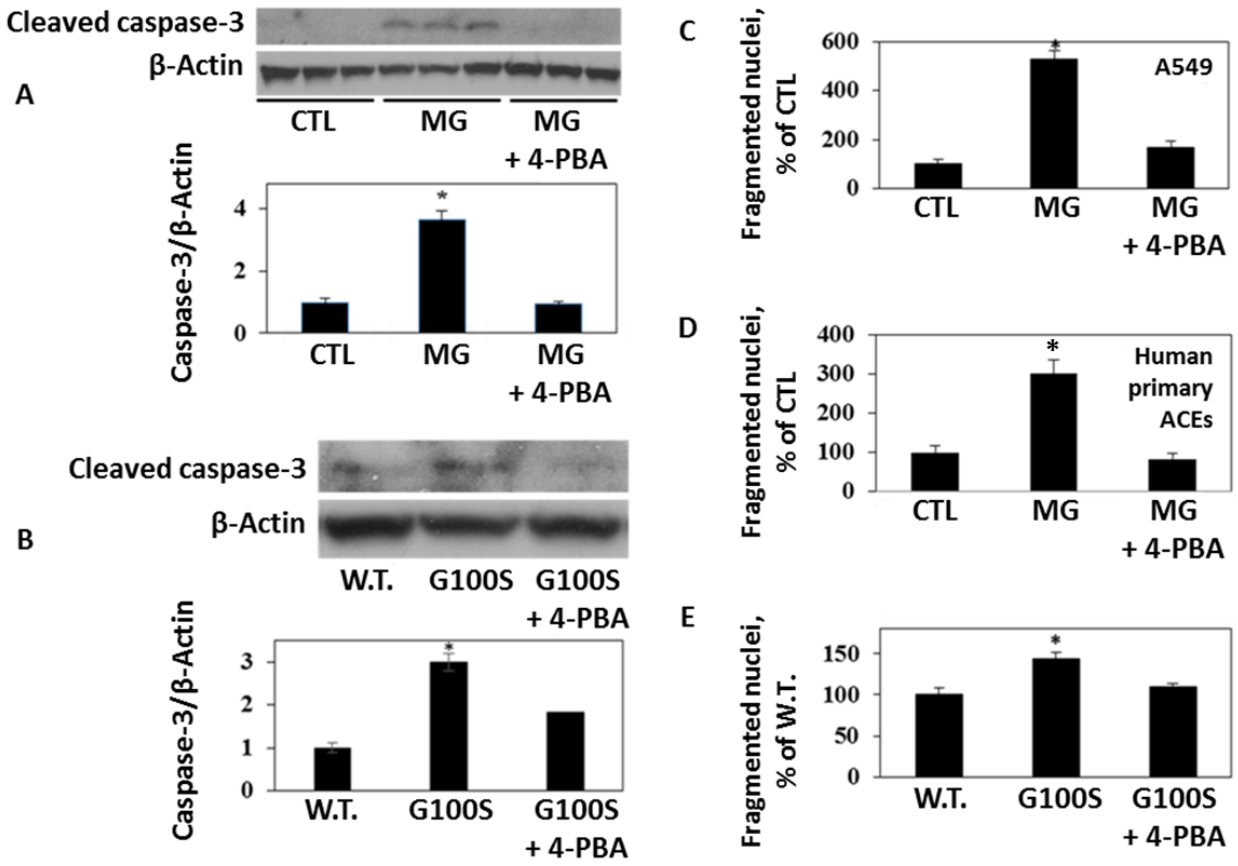


**Figure 3.6. The chemical chaperone 4-PBA prevents ER stress-induced caspase-3 activation and nuclear fragmentation in AECs.** A549 cells were challenged with MG132 (10 μM, A and C) or were transiently transfected with plasmids containing wild-type SP-C or SP-C BRICHOS domain mutant G100S (B and D) in the presence or absence of 4-PBA (5 mM) for 48 h. Cells were then harvested for Western blot detection of cleaved (active form) of caspase-3 (A and B) or measured of fragmented nuclei (C and D). Bars represent the mean ± S.E.M of at least three cell cultures. *=$P < 0.05$ vs. CTL (A, C, D) and *=$P < 0.05$ vs. W.T. (B and E), all by ANOVA and Student-Newman-Keul's multiple comparison test.

***Inhibition of UPR activation can prevent ER stress-induced cathepsin D activation or ACE-2 downregulation***

To further investigate which UPR pathway is involved in regulation of the angiotensin system, we used the siRNAs against each target sensor in order to block the activity of each pathway. Efficiency of the knockdown was assessed using Western blot (Fig. 3.7). Figure 3.8 demonstrated that inhibition of ATF6 or IRE1 pathway by knockdown of ATF6 or IRE1 pathway using antisense oligonucleotides showed a potential to prevent MG132- or SP-C mutation-induced cathepsin D activation of A549 cells. In addition, knockdown of ATF6 or IRE1 pathway using antisense oligonucleotides showed significant effect on preventing of ER stress-induced ACE-2 downregulation (Fig. 3.9). On the other hand, blocking of PERK pathway by antisense nucleotides did not show effect on diminishing of ER stress-induced cathepsin D activation or ACE-2 downregulation (Fig. 3.8 and 3.9).

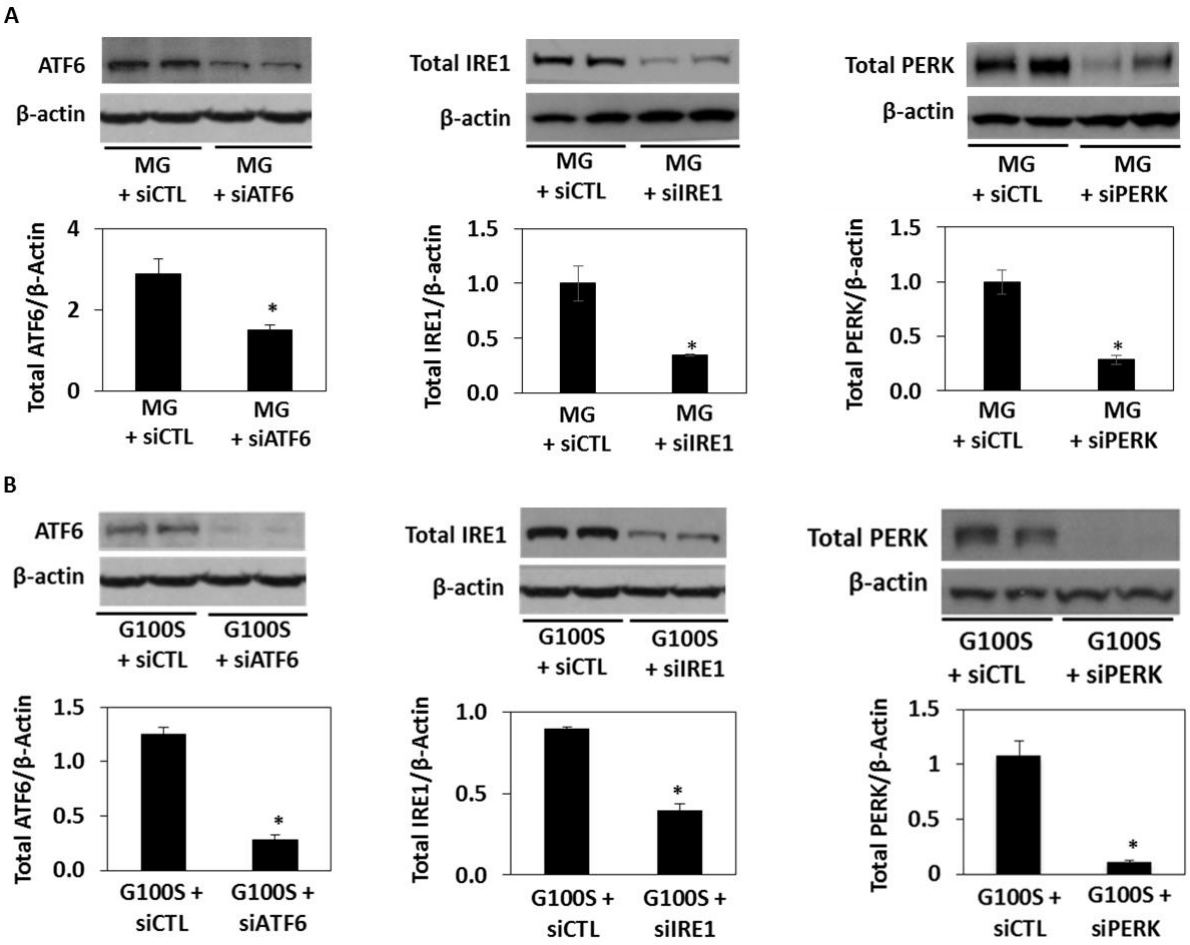


**Figure 3.7. Knockdown efficiency of UPR pathways using antisense oligonucleotides.** A549 cells were challenged with either siRNAs against UPR target sensors (siATF6, siIRE1, siPERK) or scramble siRNAs (siCTL) for 24 h then were treated with MG132 (10 μM, A) for 24 h or were transfected with plasmids containing wild-type SP-C or SP-C BRICHOS domain mutant G100S for 48 h (B). Western blotting was used to assess the efficiency of the knockdown. β-actin was used as loading control. *=P < 0.05 vs. MG + siCTL (A) or vs. G100S + siCTL by Student's *t*-test (B).

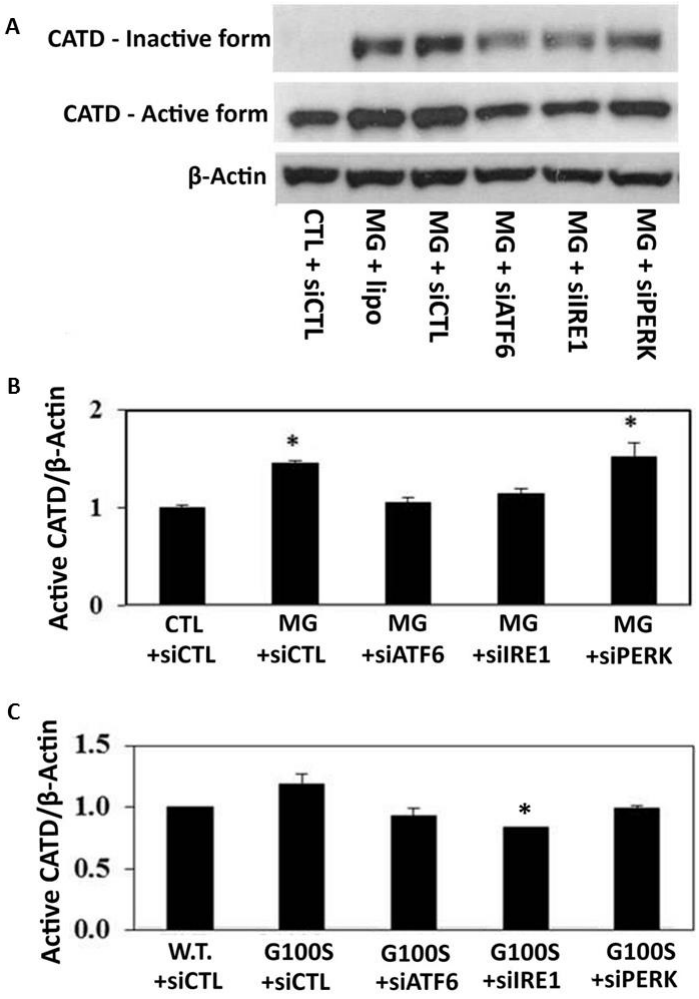


**Figure 3.8. Knockdown of UPR pathways using antisense oligonucleotides can prevent the ER stress-induced cathepsin D upregulation.** A549 cells were challenged with either siRNAs against UPR target sensors (siATF6, siIRE1, siPERK) or scramble siRNAs (siCTL) for 24 h then were treated with MG132 (10 µM, A and B) for 24 h or were transfected with plasmids containing wild-type SP-C or SP-C BRICHOS domain mutant G100S for 48 h (panel C). Western blotting was used to detect cathepsin D protein (active form) from whole-cell lysates. β-actin was used as loading control. *=P < 0.01 vs. CTL + siCTL using ANOVA and Student-Newman-Keul's multiple comparison test (B), and *=P < 0.05 vs. G100S + siCTL by Student's *t*-test (C).

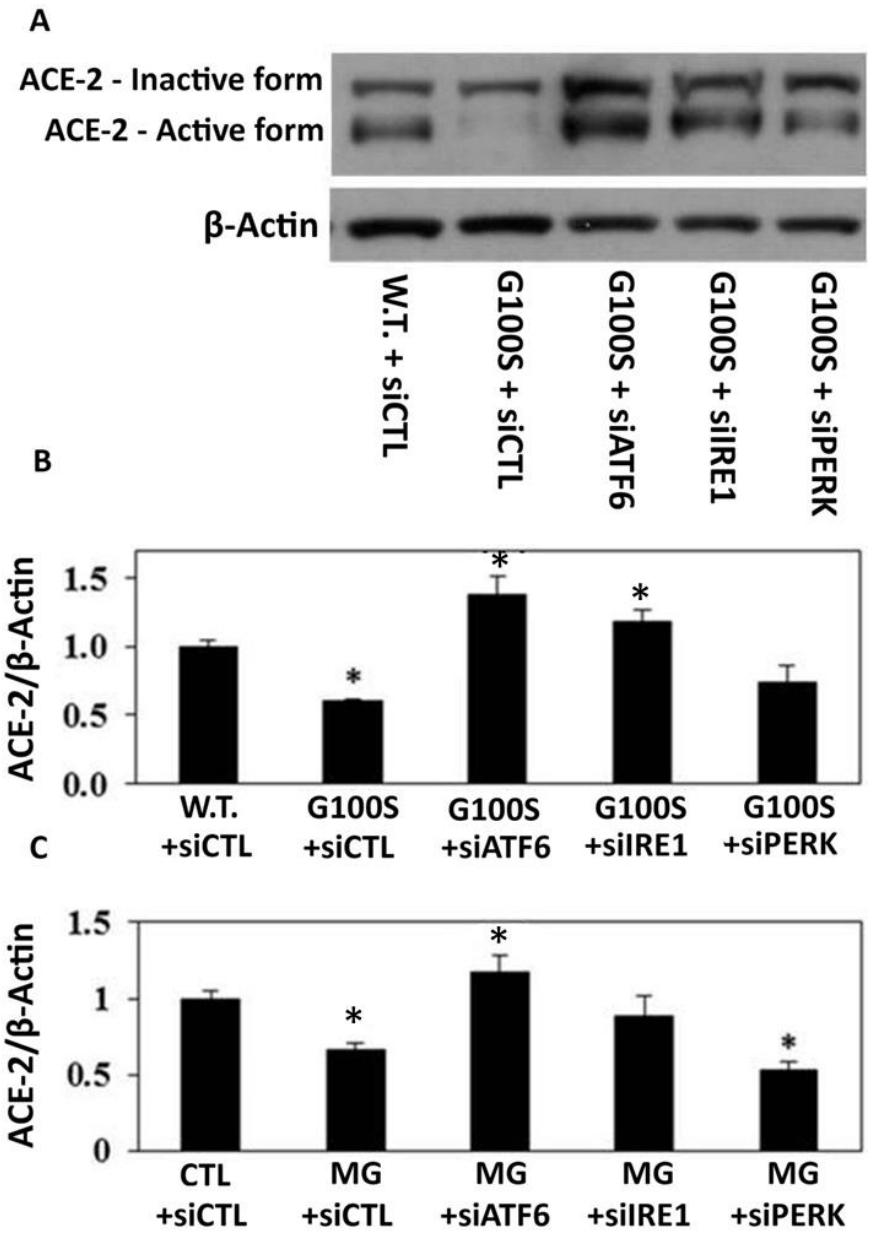


**Figure 3.9. Blockade of UPR pathways by antisense oligonucleotides can prevent the ER stress-induced decrease of ACE-2 protein.** A549 cells were challenged with either siRNAs against UPR target sensors (siATF6, siIRE1, siPERK) or scramble siRNAs (siCTL) for 24 h and then were transfected with plasmids containing wild-type SP-C or SP-C BRICHOS domain mutant G100S (panel A and B) for 48 h or were incubated with MG132 (10 μM, panel C) for 24 h. Whole-cell lysates were harvested for detection of ACE-2 protein (inactive and active forms) by Western blotting. Anti β-actin antibody was used as loading control. *=$P < 0.05$ vs. W.T. + siCTL (B) or vs. CTL + siCTL (C), all by ANOVA and Student-Newman-Keul's multiple comparison test.

***Inhibition of UPR activation can prevent ER stress-induced ADAM17/TACE activation***

Consistent with these data above, knockdown of ATF6 or IRE1 pathway using antisense oligonucleotides, compared to PERK pathway, appeared to have more effect on preventing of SP-C mutation-induced TACE activation in A549 cells (Fig. 3.10A).

Recent studies have reported the findings of various potent and selective inhibitors for IRE1 and PERK (1, 3). Among those is 4μ8C, which has been demonstrated to be a selective blocker of IRE1 that can inactivate the Xbp1 splicing and IRE1-mediated mRNA degradation in MEF and HEK-293T cells (3). Furthermore, Atkins and colleagues have identified GSK2656157, an ATP-competitive inhibitor, as a potent blocker of PERK which has been shown the ability to prevent ER stress-induced PERK and eIF2α phosphorylation in human pancreatic adenocarcinoma BxPC3 cells (1). Figure 3.10B showed that 4μ8C can prevent the TACE upregulation in response to MG-induced ER stress while GSK2656157 did not show the similar effect.

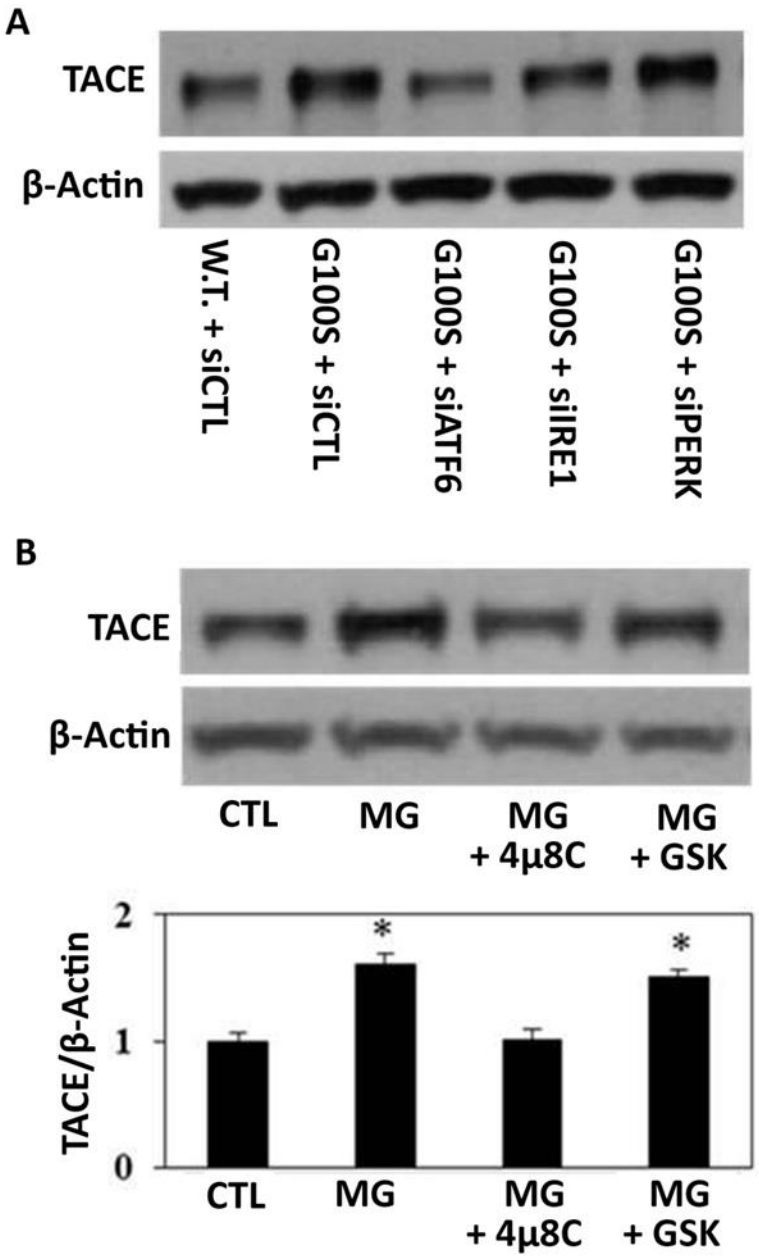


**Figure 3.10. Inhibition of UPR pathways can prevent ER stress-induced ADAM17/TACE activation of AECs.** In panel A, human A549 cell line was challenged with either siRNAs against UPR target sensors (siATF6, siIRE1, siPERK) or scramble siRNAs (siCTL) for 24 h then was transfected with plasmids containing wild-type SP-C or SP-C BRICHOS domain mutant G100S for 48 h. In panel B, A549 cells were incubated with IRE1 blocker (4μ8C, 50 μM) or PERK blocker (GSK2656157, 1 μM) for 1 h before treated with MG132 (10 μM) for 24 h. Whole-cell lysates were harvested for detection of ADAM17/TACE protein by Western blotting. β-actin was used as loading control. *=$P < 0.05$ vs. CTL (B) by ANOVA and Student-Newman-Keul's multiple comparison test.

**DISCUSSION**

A variety of studies have documented roles for apoptosis of AECs in acute lung injury, COPD and pulmonary fibrosis (15). Recent studies have reported that mutations in the BRICHOS domain of the SP-C, which is exclusively synthesized by alveolar epithelial type II cells, can result in protein misfolding, accumulation, induction of ER stress and apoptosis of AECs (8, 20). However, chronic ER stress and UPR activation have also been observed in both sporadic and familial IPF, despite the absences of SP-C mutations (8). Taken together, multiple reports implicated that ER stress and UPR activation have a prominent role in the pathogenesis of IPF (27).

Several lines of evidence have established that angiotensin system plays important role in the pathogenesis of lung fibrosis, including the apoptosis of lung AECs (29). Alveolar cells treated with apoptosis inducers such as bleomycin or endogenous toxins (Fas ligand, TNFα) showed an increase in AGT mRNA, AGT protein and the derived enzyme octapeptide ANGII (34, 36). ANGII is known as pro-apoptotic and is required for the apoptosis of epithelial cells in many organs, including the liver, pancreas and lungs (30). An earlier study from our laboratory reported that bleomycin-induced apoptosis of AECs requires the conversion of AGT to ANGII, a process that requires the proteolytic activity of cathepsin D (14). Additionally, our recently published work showed that ER stress inducer MG132 upregulated cathepsin D activation in AECs (33). The finding herein showed an increase in the level of cathepsin D in response to ER stress can be reduced by chemical chaperone 4-PBA, or by knockdown of the ATF6 or IRE1 pathway. The resulting reduction of cathepsin D (or an increase in ACE-2) would reduce

apoptosis by reducing the ANGII/ANG1-7 ratio as discussed earlier (33). The mechanisms by which ATF6 or IRE1 regulates cathepsin D activation would be an interesting topic for future investigation.

Recent reports from our laboratory demonstrated that ACE-2 is a protective protein, which inhibits apoptosis through its abilities of balancing the pro-apoptotic ANGII and its anti-apoptotic degradation product ANG1-7 (31). In this study, we suggest that chemical chaperone 4-PBA could not significantly prevent the decrease ACE-2 when challenged with MG132 or G100S SP-C mutation, but ATF6 or IRE1 has the potential to inhibit this downregulation of ACE-2. Moreover, studies of cultured human airway epithelial cells have suggested that shedding of ACE-2 ectodomain plays important role in SARS infection or inflammation (21, 23, 33). Our previous study has reported that ACE-2 ectodomain shedding enzyme ADAM17/TACE plays a role in the G100S-induced loss of ACE-2 (33). In addition, it was also demonstrated in our study that TAPI2, an ADAM17/TACE inhibitor, could prevent MG132- or clastocystin-induced cathepsin D activation of A549 cells (33). The findings herein demonstrate that either MG132 or G100S SP-C mutation upregulated the activation of ADAM17/TACE protein, and chemical chaperone 4-PBA could prevent the G100S-induced TACE activation of alveolar epithelial A549 cells. It is also suggested that blocking of ATF6 or IRE1 pathway by knockdown of antisense oligonucleotides or by chemical inhibitor (4μ8C) showed potential to prevent the ER stress-induced TACE activation. These results suggested that the ER stress and UPR activation might regulate the apoptosis of AECs via angiotensin signaling mechanisms that involve the ADAM17/TACE induction, and either ATF6- or IRE1-induced TACE activation has the potential to explain the induction of apoptosis of AECs by ER stress.

Previous work from our laboratory has demonstrated that the autocrine ANGII/ANG1-7 system regulates apoptosis of AECs in response to ER stress induced by either chemical proteasome inhibitor (MG132) or by SP-C BRICHOS domain mutation (G100S, 33). In that study, we reported that either the protective anti-apoptotic peptide ANG1-7 or nonselective ANGII receptor blocker saralasin could significantly prevent the ER stress-induced apoptosis of alveolar epithelial cells by inhibiting the caspase activation and nuclear fragmentation. The present study showed that UPR blockers inhibited the ER stress-induced apoptosis of AECs. Taken together, our results support the concept of UPR-modulated changes in the autocrine ANGII/ANG1-7 system in the regulation of ER stress-induced apoptosis of AECs.

Our recent studies found that anti-apoptotic ANG1-7 could inhibit JNK phosphorylation in AECs challenged with either apoptotic agent bleomycin or ANGII (30). Additionally, inhibition of ANG1-7 receptor mas by A779 or antisense nucleotides against mas mRNA showed an increase in the level of phosphorylated JNK and cleaved caspase-3, thus enhancing bleomycin-induced apoptosis of AECs (31, 33). On the other hand, ANGII-induced apoptosis of AECs is mediated by ANG receptor, which also could be blocked by AT1-selective ANG blockers (losartan) or an ANG-nonselective blocker (saralasin). In the light of our recent study showing that the autocrine ANGII/ANG1-7 system is involved in the ER stress-induced apoptosis of AECs (33), it is suggested here that ER stress and UPR activation might potentially modulate the activity of both angiotensin axes through the mechanisms that are yet to be elucidated.

In summary, this study demonstrates that ER stress induced by either proteasome inhibitor MG132 or by SP-C BRICHOS domain mutation G100S could lead to the activation of all

three conical pathways of the UPR. Blockade of UPR pathways could lead to a decrease in the ANGII-producing pathway cathepsin D and ACE-2 ectodomain shedding protein ADAM17/TACE or a decrease in ANGII-degrading pathway ACE-2. We anticipate that ER stress-induced apoptosis of human AECs mediated by UPR pathways that modulate the autocrine ANGII/ANG1-7 system of these cells, and suggest that understanding the mechanisms by which ER stress and UPR regulate the angiotensin system will support to understand the apoptosis of AECs, thus provide potential treatments of fibrotic lung disease.

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## CHAPTER 4

## REGULATION OF ANGIOTENSIN-CONVERTING ENZYME 2 BY HYPEROXIA AND HYPOXIA IN HUMAN FETAL LUNG FIBROBLASTS[1]

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[1] The work described in this chapter was published as the following manuscripts:

Tarek Mohamed, Hang Nguyen, Amal Abdul-Hafez, Vinh X. Dang, My-Trang Dang, Ira H. Gewolb and Bruce D. Uhal. Prior hypoxia prevents downregulation of ACE-2 by hyperoxia in human fetal lung fibroblasts. *Experimental Lung Research* 42(3): 121-130, 2016.

Chinyere I. Oarhe, Vinh Dang, MyTrang Dang, Hang Nguyen, Indiwari Gopallawa, Ira Gewolb and Bruce D. Uhal. Hyperoxia downregulates ACE-2 in human fetal lung fibroblasts. *Pediatric Research* 77(5): 656-62, 2015.

**ABSTRACT**

Hyperoxia has been known as a fibrotic agent in neonatal lung disease. Angiotensin converting enzyme-2 (ACE-2), which degrades proapoptotic ANGII into anti-apoptotic ANG1-7, is protective and downregulated in adult human and experimental lung fibrosis. However, the role of ACE-2 in response to hyperoxic exposure of human fetal fibroblasts has not yet been evaluated. In this study, human fetal fibroblasts cell line, IMR-90 were exposed to hyperoxia (95% $O_2$/5% $CO_2$) or normoxia (21% $O_2$/5% $CO_2$). Furthermore, IMR-90 cells were also incubated in hypoxia (1% $O_2$/99% $N_2$) followed by hyperoxia (95% $O_2$/5% $CO_2$) or normoxia (21% $O_2$/5% $CO_2$). Cells were assessed for ACE-2 and ADAM17/TACE mRNA and protein expression. Epithelial-to-mesenchymal transition (EMT) markers such as α-SMA, N-cadherin and β-catenin were also measured. The report herein demonstrated that ACE-2 is expressed in human fetal fibroblasts in response to hyperoxia, and is downregulated at the protein level, but not mRNA level. It suggests that hypoxia prior to hyperoxic exposure might be protective to fetal lung fibroblasts by upregulating ACE-2 and downregulating ACE-2 ectodomain shedding enzyme, ADAM17/TACE. Biomarkers of EMT of human fetal fibroblasts were also shown to be downregulated in response to hypoxia prior to hyperoxic exposure.

## INTRODUCTION

Children born prematurely often exhibit reduced pulmonary function and lung capacity (26). Supplemental oxygen, which is often used to treat premature infants with pulmonary insufficiency, has been implicated in the development of bronchopulmonary dysplasia (BPD, 16). Hyperoxia has been known to cause chronic fibrotic lung disease in neonates (16). Prolonged exposure of neonatal mice to hyperoxia led to impaired alveolarization and increased lung fibrosis that is similar to human BPD (16).

Hypoxia, which is defined as the failure of oxygenation at the tissue level, can induce lung injury and may contribute to BPD (14). There is a strong association between hypoxia and subsequent chronic lung disease (14). Ratner and colleagues have suggested that cycling hypoxia with hyperoxia can exacerbate lung injury (17). They also demonstrated that intermittent hypoxia without hyperoxic exposure did not show any change in the markers of oxidative stress and lung injury (17).

EMT is a process in which epithelial cells lose their phenotype, acquire fibroblast-like properties, reduced cell adhesion and increased motility (19). It is integral to development of organs, wound healing and cancer progression (9). EMT also has been reported to contribute to fibrotic pathogenesis of many organs, including kidney and lung, by increasing the collagen accumulation and matrix deposition (20). Recent findings suggested that AECs themselves can give rise to fibroblast/myofibroblasts through the process of EMT (27). Myofibroblasts, key feature cells in IPF, have been suggested to arise from resident lung fibroblasts or from AECs that have undergone EMT (27).

Earlier work from our laboratory showed that angiotensin-converting enzyme 2 (ACE-2), which degrades octapeptide ANGII to form heptapeptide ANG1-7, protects against lung fibrosis by limiting the accumulation of ANGII (24). ACE-2 mRNA, protein and enzymatic activity were significantly decreased in human and experimental lung fibrosis induced by bleomycin (24). Blockade of ACE-2 by gene silencing or by competitive inhibitor DX600 not only significantly increased ANGII level, but also enhanced collagen accumulation in mice exposed to bleomycin (24). Additionally, other authors have reported that administration of purified recombinant ACE-2 attenuated bleomycin-induced collagen deposition in mice models of pulmonary fibrosis (22).

Li and colleagues have identified ACE-2 as a cellular receptor of severe acute respiratory syndrome coronavirus (SARS-CoV, 11). Haga *et al.* recently reported the role of a disintegrin and metalloprotease (ADAM) family member TNFα-converting enzyme (TACE) that is required for the viral entry (5). Previous studies showed that TACE is involved in the process of shedding the ectodomain of ACE-2, which releases its soluble forms for multiple functions (7). It is shown that TACE-dependent shedding of ACE-2 ectodomain is completely dependent on the binding of SARS-S protein to ACE-2 (5). Blocking of the shedding activity can be achieved by TACE inhibitors such as TAPI-0 or TAPI-2. It is now known that ADAM17/TACE is involved in the cleavage and release of ACE-2 ectodomain in several tissues (16).

This study tested the hypothesis that ACE-2 might be downregulated by hyperoxic exposure in human fetal fibroblasts. It is also hypothesized that hypoxia prior to hyperoxic

exposure might be protective to fetal lung fibroblasts by preventing ACE-2 downregulation, and activating ACE-2 ectodomain shedding enzyme ADAM17/TACE.

## MATERIALS AND METHODS

### *Reagents and materials*

Antibodies against ACE-2 and ADAM17/TACE were obtained from Abcam (Cambridge, MA) and Cell Signaling Technology (Danvers, MA), respectively. Antibodies for EMT markers against α-SMA, N-cadherin, β-catenin, and β-actin were purchased from Cell Signaling Technology (Danvers, MA). HRP-conjugated anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Denville Scientific (Holliston, MA) or BioRad Co. (Melville, NY).

### *Cell culture*

This study was approved by the Institutional Review Board of Michigan State University (East Lansing, MI). Hypoxia/hyperoxia gas tanks were under premixed conditions and purchased from Airgas (Lansing, MI). The hypoxia/hyperoxia chamber was flushed with the appropriate treatment gas, at a flow of 3 L per min (LPM) for 45-60 min (14). The flow was then decreased to 0.5 LPM until the oxygen analyzer indicated that the desired oxygen level had been reached (14). Each well of the 6-well cell culture plates was filled with 10 mL of medium to ensure no changes in solute concentration or pH after the treatment period (14).

Human fetal lung fibroblast cell line IMR-90 was purchased from ATCC (Manassas, VA) and was cultured in collagen-coated plates. When reached post-confluency, cells were exposed to hyperoxia (95% $O_2$ with 5% $CO_2$) or normoxic (21% $O_2$ with 5% $CO_2$) gas for 72 h in cell culture media supplemented with 10% FBS and antibiotics. Cells were then recovered in serum-free media for 24 h. Cell lysates were either harvested at the end of 72 h period or after 24 h of recovery. For hypoxia preceding hyperoxia studies, cells were exposed to hypoxia (1% $O_2$ with 99% $N_2$) for 24 h in 5% FBS, bicarbonate-free MEM with HEPES buffer followed by hyperoxia (95% $O_2$ with 5% $CO_2$) for 72 h in 5% FBS complete MEM. Cells were then either harvested for immediately or treated under "recovery phase" (21% $O_2$ with 5% $CO_2$) in serum-free media (14).

***Western blotting***

IMR-90 cell lysates were collected in ice-cold protein lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate and protease inhibitor cocktail (Complete Mini, Roche, Nutley, NJ)). Cell suspensions were centrifuged at 11000g for 15 minutes at 4$^{o}$C and supernatants were collected for protein detection. Protein samples were ran on 7.5–12% SDS gradient polyacrylamide gels and transferred to PVDF membranes. Membranes were incubated in 5% nonfat dry milk buffer for 1 hour at room temperature. They were then incubated with primary antibodies at 4$^{o}$C overnight then with secondary antibodies for 1 hour at room temperature. Detection of the proteins were performed using Supersignal West Pico chemiluminescent substrate (GE Healthcare Biosciences, Pittsburgh, PA). β-actin antibodies were used as loading control.

***Real-time quantitative PCR***

After treatment, IMR-90 cells were extracted for total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using Superscript II reverse transcriptase and oligo $(dT)_{12-18}$ (Invitrogen). Real-time PCR was carried out with cDNA synthesized from 50 ng of total RNA, [SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA, USA)] according to the manufacturer's instructions, and 0.2 µM specific primers for human ADAM17/TACE (sense 5'-TTG GTG GTA GCA GAT CAT CG-3' and antisense 5'-CTG GGA GAG CCA ACA TAA GC-3') and GAPDH (Sense 5'-CCC CTT CAT ACC CTC ACG TA-3' and Antisense 5'-ACA AGC TTC CCG TTC TCA G-3'). The thermal profile started with 10 minutes of activation at 95°C followed by 40 cycles of denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds, extension at 72°C for 60 seconds, and ending with a dissociation curve analysis of the PCR products. Reactions were performed in the StepOnePlus Real-time PCR system instrument (Applied Biosystems). Threshold cycle (CT) data were collected using StepOne software version 2.1 (Applied Biosystems). The relative TACE/ADAM-17 expression was normalized to GADPH and calculated with the comparative CT method of $2^{-\Delta\Delta CT}$, where $\Delta CT = CT_{ACE\text{-}2} - CT_{GAPDH}$ and $\Delta\Delta CT = \Delta CT_{treatment} - \Delta CT_{control}$.

**RESULTS**

***Hyperoxia does not significantly change caspase-9 activation of human fetal lung fibrolasts***

Human fetal fibroblast cell line IMR-90 was exposed to either hyperoxic (95% $O_2$/5% $CO_2$) or normoxic (21% $O_2$/5% $CO_2$) conditions for 24 hours. At the end of 72 hours, cells were either harvested for whole-cell lysates (No recovery, Fig. 4.1, panel A), or incubated for an additional 24 hours in serum-free media (24 hour recovery, Fig. 4.1, panel B) before harvesting. Cleavage of caspase-9, one of the apoptotic markers, was measured by Western blotting. Figure 4.1 showed that there is no significant change in the level of active caspase-9 in the treatment group, compared to the control regardless of a recovery phase.

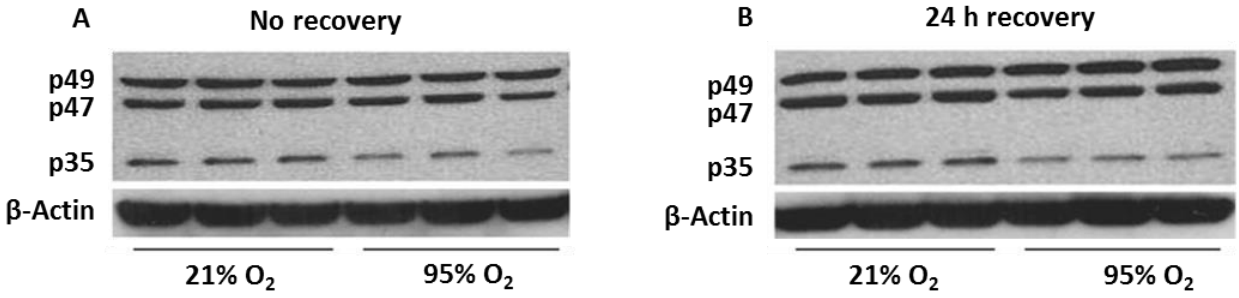


**Figure 4.1. Caspase-9 in human fetal lung fibrolasts exposed to hyperoxic or normoxic conditions.** Human fetal lung IRM90 cells were exposed to either hyperoxic (95% $O_2$/5% $CO_2$) or normoxic (21% $O_2$/5% $CO_2$) gas in 72 h, without (A) or with (B) a 24 h recovery. Whole-cell lysates were then measured for cleavage of caspase-9, with p49 and p47 are pro-caspase-9 and p35 is cleaved form. β-actin was used as loading control. Quantitation of densitometry showed no statistically significant difference in the caspase-9/β-actin ratio between the two groups.

***Hyperoxia with normoxic recovery upregulated ADAM17/TACE mRNA, but not ACE-2 mRNA***

IMR-90 cells were exposed to hyperoxic (95% $O_2$/5% $CO_2$) or normoxic (21% $O_2$/5% $CO_2$) gas in 72 hours, with an additional 24 hours for recovery. Cells were assessed for the mRNA levels of ACE-2 and ADAM17/TACE. Figure 4.2 suggested that hyperoxia with normoxic recovery in human fetal fibroblast IMR-90 cells showed no significant change in the level of ACE-2 mRNA (panel A), but significantly increased TACE mRNA (panel B).

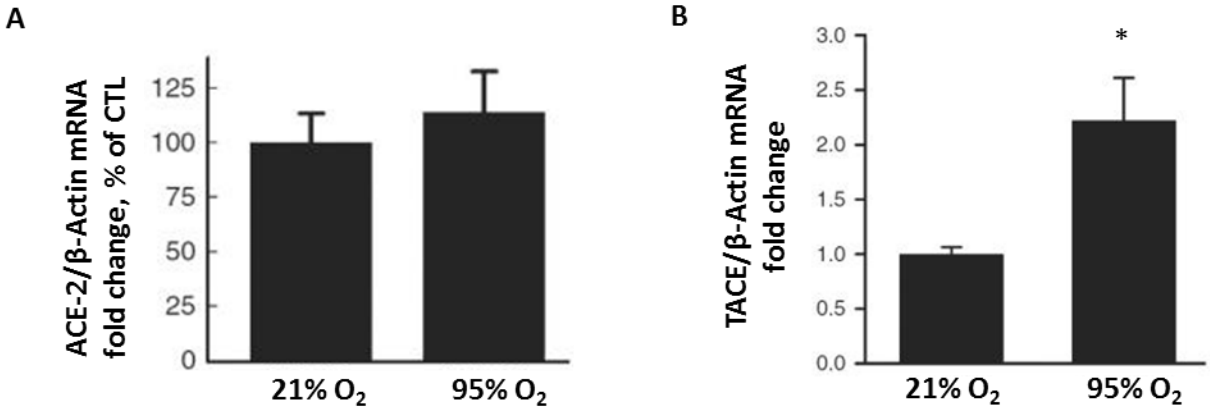


**Figure 4.2. Hyperoxia with normoxic recovery upregulates ADAM17/TACE mRNA, but not ACE-2 mRNA.** Human fetal lung IRM90 cells were exposed to either hyperoxic (95% $O_2$/5% $CO_2$) or normoxic (21% $O_2$/5% $CO_2$) gas in 72 h, with an additional 24 hour in serum-free media. Whole-cell lysates were harvested for total RNA. In panel A: quantitation of ACE-2 mRNA showed no statistically significant difference in the ACE-2/β-actin ratio between the two groups. In panel B: hyperoxia with normoxic recovery upregulated ADAM17/TACE mRNA level. Quantitation represents the mean ± S.E.M of at least three independent experiments. *=P < 0.05 vs. 21% $O_2$ by ANOVA and Student-Newman-Keul's post hoc test.

***ACE-2 protein was upregulated by hypoxia prior to hyperoxic exposure in human lung fibroblasts***

IMR90s treated with hypoxia prior to hyperoxic exposure showed an increase in the level of ACE-2 immunoreactive protein. However, there was no significant change in the level of ACE-2 if these IMR-90 cells underwent the extra 24 hour recovery period with normoxic gas after the treatment. This result clarifies the work of Oarhe *et al.*, which reported that hyperoxia downregulated ACE-2 protein in IMR-90 cells (16).

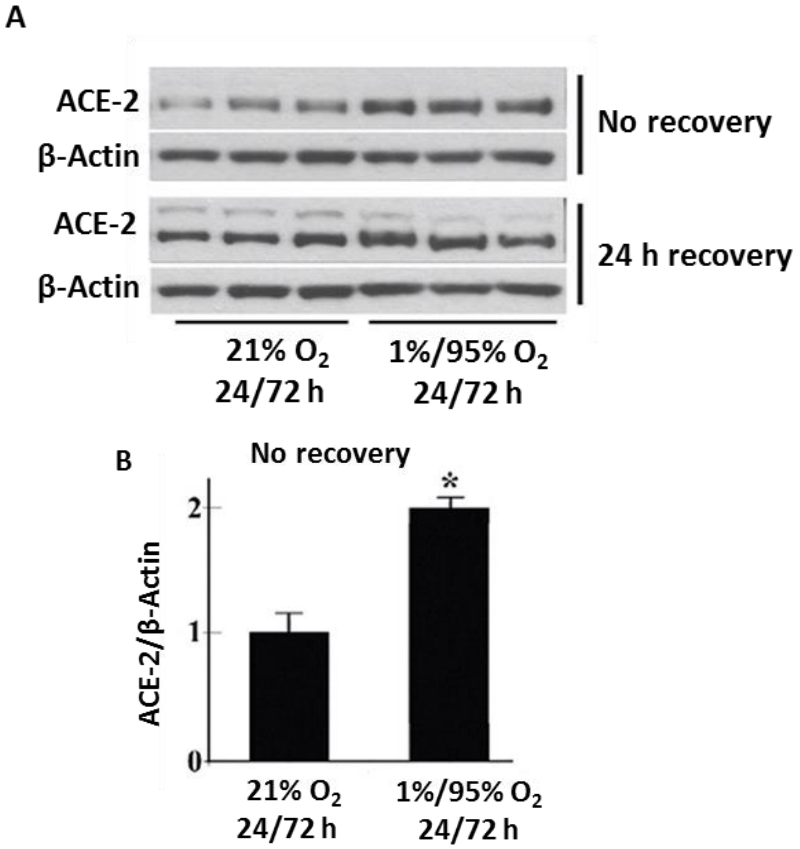


**Figure 4.3. Activation of ACE-2 by hypoxia prior to hyperoxic exposure in fetal lung fibrolasts.** Human fetal lung IRM90 cells were exposed to hypoxia for 24 h before being incubated in hyperoxic conditions for 72 h. Whole-cell lysates were used for detection of immunoreactive ACE-2 protein. β-actin was used as loading control. Quantitation represents the mean ± S.E.M of at least three independent experiments. *=P < 0.05 vs. 21% $O_2$ by ANOVA and Student-Newman-Keul's post hoc test.

***ADAM17/TACE mRNA and protein were downregulated by hypoxia prior to hyperoxic exposure in human lung fibroblasts***

Hypoxia prior to hyperoxia following by normoxic recovery reduces ADAM17/TACE protein (Fig 4.4, panel A and B). It is also shown that treatment of IMR-90s with hypoxia prior to hyperoxic exposure downregulated ADAM17/TACE mRNA level (panel C).

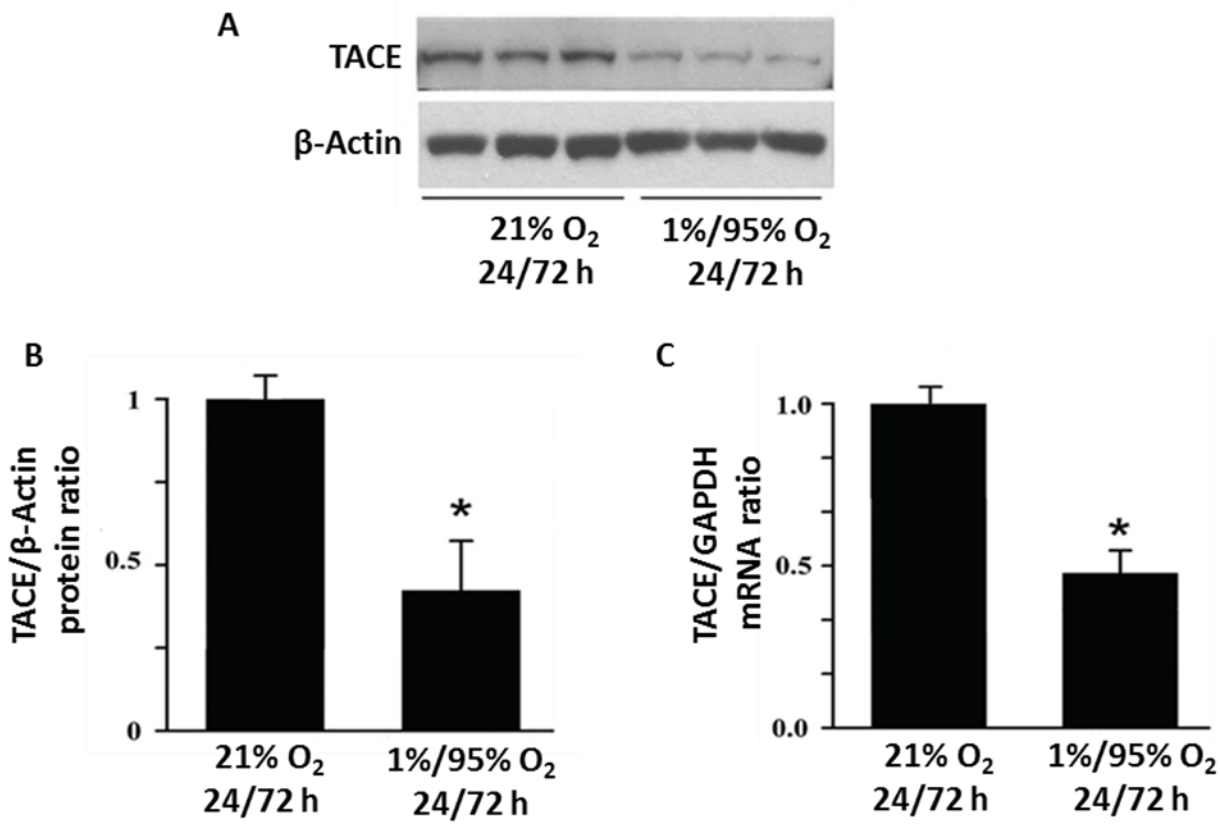


**Figure 4.4. Downregulation of ADAM17/TACE by hypoxia prior to hyperoxic exposure.** Human IMR-90 cells were exposed to hypoxia for 24 h before being exposed to hyperoxic or normoxic gas in 72h with 24 h recovery. Whole-cell lysates were used for detection of immunoreactive TACE protein (panel A and B) or for analysis of mRNA quantitation (panel C). β-actin was used as loading control (A and B), while GAPDH mRNA was used to normalize for mRNA fold change of target genes (panel C). Quantitation represents the mean ± S.E.M of at least three independent experiments. *=$P < 0.05$ vs. 21% $O_2$ by ANOVA and Student-Newman-Keul's post hoc test.

***Hypoxia prior to hyperoxic exposure downregulated* αSMA *in human lung fibroblasts***

Figure 4.5 shows that alpha-smooth muscle actin (αSMA), the standard marker for myofibroblast transition, is downregulated in fetal lung fibroblasts by hypoxia prior to hyperoxic exposure (panel A). It also suggested that IMR-90 cells challenged with hypoxia prior to hyperoxic exposure with additional normoxic recovery had significantly decrease αSMA (panel B).

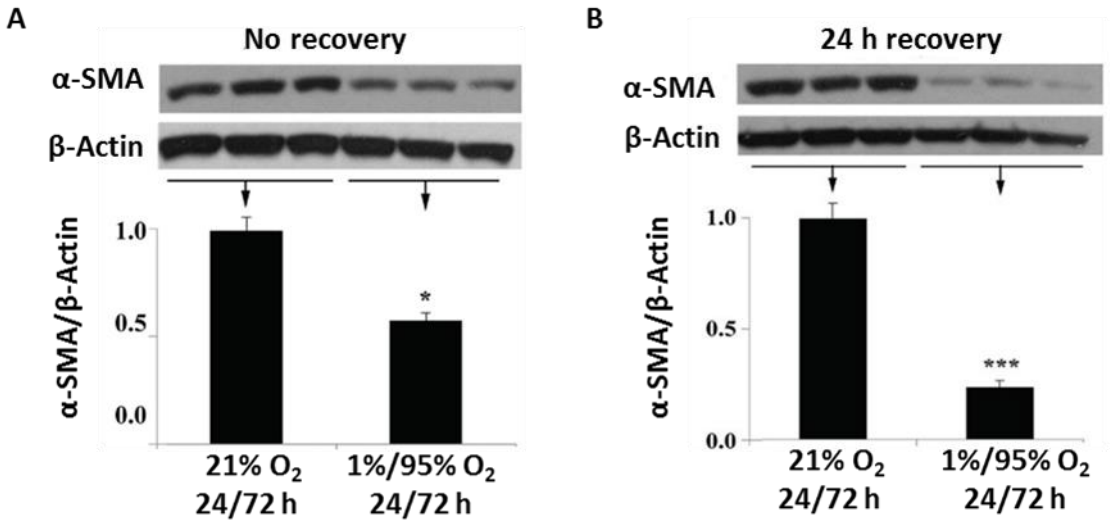


**Figure 4.5. Downregulation of α-smooth muscle actin (αSMA) by hypoxia prior to hyperoxic exposure.** Human IMR-90 cells were exposed to hypoxia for 24 h before being exposed to hyperoxic or normoxic gas in 72 h, without (A) or with (B) recovery. Whole-cell lysates were used for detection of immunoreactive αSMA protein. β-actin was used as loading control. Quantitation represents the mean ± S.E.M of at least three independent experiments. *=P < 0.05 and ***=P < 0.001 vs. 21% $O_2$ by ANOVA and Student-Newman-Keul's post hoc test.

***Hypoxia prior to hyperoxic exposure downregulated N-cadherin in human lung fibroblasts***

Cell-cell junctions are essential for the function and integrity of epithelial cells. Upon the initiation of EMT, the junction proteins are reconstructed, destabilized and/or degraded (9). During this process, epithelial cadherin (E-cadherin) is downregulated to reinforce the destabilization of adherens junction. Downregulation of E-cadherin is balanced by the increase in the expression of mesenchymal neural cadherin (N-cadherin), resulting in a "cadherin switch" that change cell adhesion (9). In figure 4.6, N-cadherin is significantly decreased in human fetal fibroblast IMR-90 cells challenged to hypoxia prior to hyperoxic gas, with or without normoxic recovery.

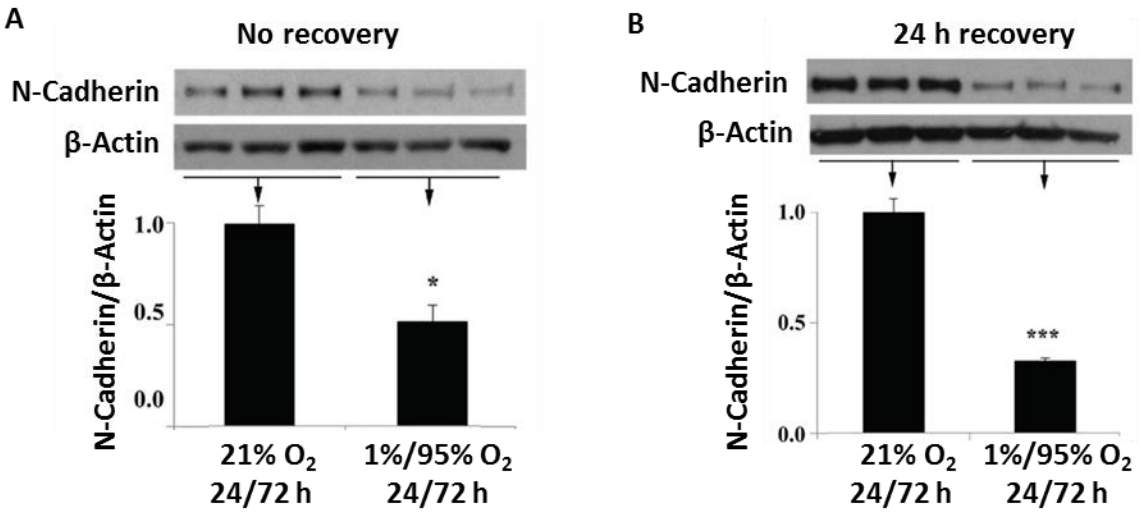


**Figure 4.6. Downregulation of N-cadherin by hypoxia prior to hyperoxic exposure.** Human IMR-90 cells were exposed to hypoxia for 24 h before being exposed to hyperoxic or normoxic gas in 72 h, without (A) or with (B) recovery. Whole-cell lysates were used for detection of immunoreactive N-cadherin protein. β-actin was used as loading control. Quantitation represents the mean ± S.E.M of at least three independent experiments. *=P < 0.05 and ***=P < 0.001 vs. 21% $O_2$ by ANOVA and Student-Newman-Keul's post hoc test.

***Hypoxia prior to hyperoxic exposure downregulated β-catenin in human lung fibroblasts***

During the destabilization of cell-cell junctions, E-cadherin is cleaved and subsequent degraded (9). β-catenin can no longer interact with E-cadherin, leading to being degraded (9). Consistent with the above results, human fetal fibroblast IMR-90 cells in response to hypoxia preceding hyperoxia showed a decrease in the level of β-catenin protein (Fig. 4.7, panel A). Similarly, hypoxia prior to hyperoxia following normoxic recovery also reduced β-catenin protein expression in human fetal fibroblasts (panel B).

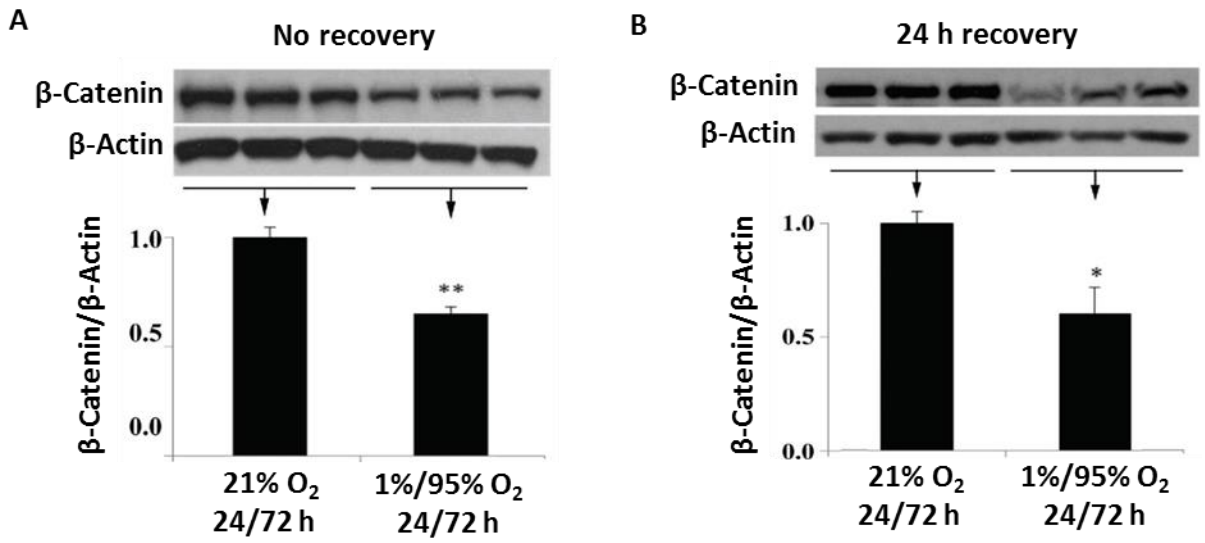


**Figure 4.7. Downregulation of β-catenin by hypoxia prior to hyperoxic exposure.** Human IMR-90 cells were exposed to hypoxia for 24 h before being exposed to hyperoxic or normoxic gas in 72 h, without (A) or with (B) recovery. Whole-cell lysates were used for detection of immunoreactive β-catenin protein. β-actin was used as loading control. Quantitation represents the mean ± S.E.M of at least three independent experiments. **=$P < 0.01$ and *=$P < 0.05$ vs. 21% $O_2$ by ANOVA and Student-Newman-Keul's post hoc test.

**DISCUSSION**

Pulmonary fibrosis has been considered as a disease of epithelial-fibroblast imbalance, which is characterized by epithelial cell damage and fibroblast/myofibroblast accumulation (25). Selman *et al.* demonstrated that pulmonary fibrosis is a process that involves in the formation of fibroblast/myofibroblast, accumulation of extracellular matrix (ECM) and abnormal wound healing (19). Developing lungs normally have myofibroblasts along the terminal airways (14). However, in injured lungs, myofibroblasts were generated significantly through a process mediated by transforming growth factor (TGF-β), which negatively regulates the branching and septation process of lung development (1, 21). Recent studies reported that treatment of lung epithelial cells and primary ACEs with TGF-β can induce EMT, which contribute to the fibroblast population of these cells (27).

EMT is a physiological process in which epithelial cells lose their phenotype, acquire fibroblast-like characteristics, reduce cell adhesion and increase cell motility (19). It is integral to the development of many tissues and organs (15), and is suggested as a source of myofibroblasts in IPF lungs (19). Hallmarks of EMT include the loss of E-cadherin and cytokeratin, as well as the increase of mesenchymal markers, such as vimentin, N-Cadherin, β-catenin and α-smooth muscle actin (αSMA) (3). The data herein showed that these EMT markers (N-Cadherin, αSMA, β-catenin) of human fetal fibroblasts were downregulated in response to hypoxia preceding hyperoxia, suggesting that hypoxia prior to hyperoxia might a protective mechanism (14). Further studies will need to examine whether the alveolar epithelial

cells response in similar “protective” manner to hypoxia preceding hyperoxia, and the signaling mechanism(s) that regulates this process will also need to be elucidated.

Prematurely born infants who received prolonged mechanical ventilation and oxygen supplementation often develop bronchopulmonary dysplasia (BDP) (1). It is believed that hyperoxia in neonatal mice and mouse lung cells disrupt TGF-β/bone morphogenetic protein (BMP) signaling pathways that direct lung development. Recent studies reported that hyperoxia upregulates the expression of total collagen, p-ERK and αSMA in human lung fibroblasts (10). It also demonstrated that angiotensin II (ANGII) is a proapoptotic and profibrotic factor in various organs including the heart, kidney and liver (12). Lang *et al.* suggested that hyperoxia-induced ANGII mediates the human lung fibroblast proliferation and stimulates collagen accumulation, which can be inhibited by losartan - an ANGII type 1 receptor ($AT_1R$) blocker (10). Moreover, emerging evidence revealed that neonatal hyperoxia enhanced fibrotic disease by increasing epithelial injury in adult mice receiving bleomycin (26). Taken together, understanding how hyperoxia modulates epithelial cells in response to lung injury agents could lead to a new finding for treatment of lung diseases.

Various studies have shown that ACE-2, an ANGII-degrading protein, is protective and is downregulated in the lungs of patients with IPF (13). Imai *et al.* identified that recombinant ACE-2 can protect mice from severe acute lung injury (6). Soluble form of ACE-2, a product of ACE-2 shedding, has been shown to block the binding of the severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein to its receptor (8). Current trials for acute lung injury (GlaxoSmithKline, pipeline drug GSK2586881) with a preparation of recombinant human ACE-2

(rhACE-2) strongly support the role of ACE-2 in administration of lung injury. The study herein reported that hyperoxic exposure with normoxic recovery of human fetal fibroblasts did not significantly change ACE-2 mRNA level, but caused a drastically decrease in the levels of ACE-2 immunoreactive protein and enzymatic activity (16). It suggested that hyperoxia-induced ACE-2 reduction occurred at the protein level, but not the RNA level, through a mechanism yet to be identified.

ACE-2 protein has a catalytically active domain that undergoes shedding, which results in the proteolytic release of its soluble form(s) (7). This process is proved to be mediated by ADAM17/TACE, a metalloprotease required for the processing and release of TNFα as well as other cell surface proteins (8, 18). Haga *et al.* demonstrated that TAPI-2, a TACE inhibitor, prevented the SARS-CoV infection by blocking the shedding of ACE-2 (5). Our study reported that hyperoxia following normoxic recovery of human fetal fibroblasts IMR-90 induced the increase of ADAM17/TACE mRNA and protein, which can be prevented by using TACE blocker TAPI-2 (16). Moreover, the above result also demonstrated that hypoxia prior to hyperoxic exposure with normoxic recovery downregulated the ADAM17/TACE mRNA and protein level (14). Therefore, understanding the mechanisms by which hyperoxia/hypoxia regulates ADAM17/TACE will be an interesting topic for future investigations.

In summary, this study showed that ACE-2 is expressed not only by lung epithelial cells, but also by fetal lung fibroblasts. Hyperoxic exposure of human fetal lung fibroblasts led to decreased ACE-2 protein and enzyme activity, but not mRNA. We also demonstrated that hypoxia prior to hyperoxia with normoxic recovery upregulated ACE-2 protein, but

downregulates ADAM17/TACE mRNA and protein, as well as α-SMA, β-catenin and N-cadherin proteins. Since ACE-2 plays important role in the cell survival, further investigation on its functions in human fetal fibroblasts might explain its role in neonatal lung injury and pulmonary viral infection.

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# CHAPTER 5

# CONCLUSIONS

**CONCLUSIONS**

Idiopathic pulmonary fibrosis (IPF) is a progressive and relentless scarring of the lung with unknown etiology (5). It is a lethal interstitial lung disease (5). Recent studies have hypothesized that apoptosis of alveolar epithelial cells (AECs) is a critical feature of lung fibrosis (8). It is suggested that IPF is the result of abnormal wound healing process which consists of persistent injury and apoptosis to AECs leading to aberrant fibroblast proliferation and accumulation of collagen. Additionally, mutations in the BRICHOS domain of the SP-C protein, which is exclusively secreted by type II AECs, have been found to result in the accumulation of misfolded proteins resulting in ER stress. Recent studies have reported that ER stress and UPR markers are found in the alveolar epithelium in lung biopsies from patients with familial interstitial pneumonia even without SP-C mutations (6). Taken together, it suggested that ER stress and UPR activation may be important in the pathogenesis of IPF.

Earlier work from our laboratory reported that apoptosis of AECs in response to endogenous or xenobiotic agents was regulated by the autocrine generation of ANGII and its counterregulatory peptide ANG1-7. ACE-2 was shown to be protective against lung fibrogenesis by limiting the local accumulation of the profibrotic peptide ANGII (3). ANG1-7 could inhibit JNK phosphorylation in AECs challenged with bleomycin or ANGII. Blocking of ANG1-7 receptor *mas* by A779 or antisense nucleotides against *mas* mRNA increased JNK phosphorylation and caspase-3 activation, thus enhancing bleomycin-induced apoptosis of AECs (7). Additionally, ANGII-induced apoptosis of AECs is mediated by the ANG receptor, which also could be blocked by AT1-selective ANG blockers (losartan) or an ANG-nonselective blocker (saralasin, 7).

Studies showed that ER stress induced by either proteasome inhibitor MG132 or SP-C BRICHOS domain mutation G100S of AECs activated ANGII-producing pathways (cathepsin D) and downregulated ANGII-degrading pathways (ACE-2). Furthermore, we demonstrated that non-selective angiotensin receptor blocker saralasin or anti-apoptotic peptide ANG1-7 can prevent ER stress-induced apoptosis of AECs by inhibiting caspase activation, mitochondrial cytochrome *c* release and nuclear fragmentation (9). Additionally, this inhibition of ANG1-7 was prevented by the specific *mas* antagonist A779. The data herein demonstrated that ER stress-induced apoptosis of AECs is mediated by the autocrine ANGII/ANG1-7 system. It also suggested that administration of ACE-2 or its enzymatic product ANG1-7 may hold potential for the management of ER stress-induced fibrotic lung disease (9).

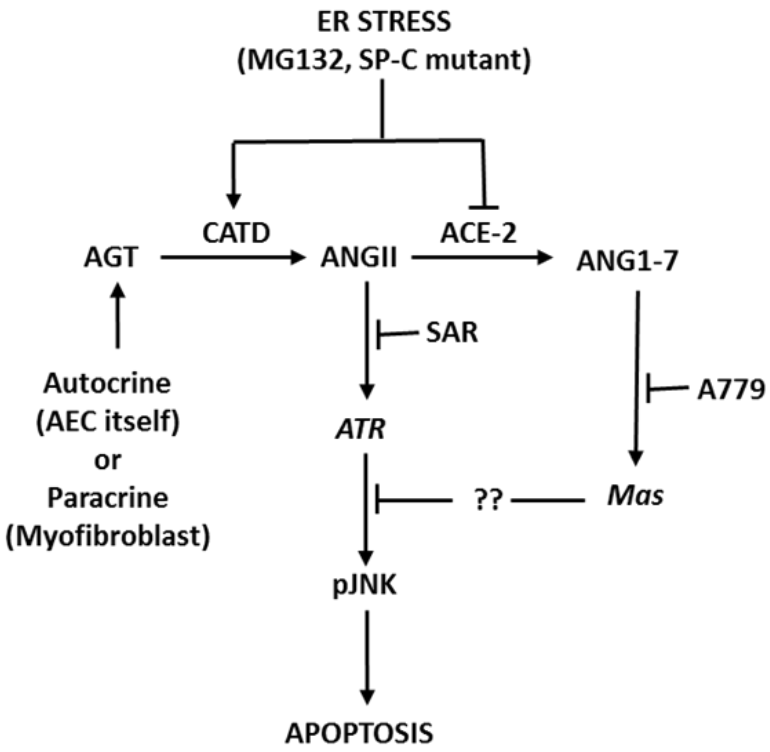


**Figure 5.1. Roles of the autocrine angiotensin system in ER stress-induced apoptosis of AECs.** ER stress induced by proteasome inhibitor (MG132) or by SP-C BRICHOS domain mutant (G100S) upregulates cathepsin D (CatD), an aspartyl enzyme that cleaves angiotensinogen (AGT) to form angiotensin II (ANGII). MG132- or SP-C mutation-induced ER stress also downregulates ACE-2, which cleaves ANGII to produce angiotensin 1-7 (ANG1-7). ER stress-induced apoptosis of AECs in response to either MG132 or the SP-C mutant G100S can be inhibited by either ANG receptor blockade (saralasin) or by administration of ANG1–7. Blocking of the ANG1-7 activity through antagonist *mas* with A779 increases ANGII receptor-mediated JNK phosphorylation by unknown mechanisms (??) that are currently under investigation.

The studies of this dissertation demonstrated that MG132- or SP-C mutation-induced ER stress activates all three canonical pathways of the UPR: ATF6, IRE1/XBP1 and PERK/eIF2α. Blockade of the UPR pathways by chemical chaperone 4-PBA led to a decrease in the ANGII-producing arm (cathepsin D and ACE-2 ectodomain shedding protein ADAM17/TACE) or a increase in ANGII-degrading arm (ACE-2). Knockdown of each UPR arm by specific antisense oligonucleotides against each sensor showed that ATF6 or IRE1 has a potential to prevent ER

stress-induced cathepsin D activation of human AECs. Additionally, knockdown of ATF6 or IRE1 pathway using antisense oligonucleotide show significant effect on inhibiting of ER stress-induced ACE-2 downregulation. Inhibition of PERK pathway by antisense nucleotides, however, did not show any significant effect on the regulation of cathepsin D or ACE-2 protein. The data herein suggest that ER stress-induced apoptosis of human AECs is mediated by UPR pathways which regulate both angiotensin axes through the mechanisms that are yet to be elucidated (Fig 5.2).

ER stress is a common event in the pathogenesis of a variety of diseases. Because ER stress-induced UPR activation has both pro- and anti-survival effects on cells, manipulation of the UPR pathways could lead to important therapeutic targets for modulating ER stress and associated diseases – including IPF (10). Available compounds to inhibit UPR activity, especially PERK and IRE1, have demonstrated potential for the treatment of numerous diseases (2). Small compounds that interact with PERK's kinase domain and IRE1α's kinase and RNAse domain, (such as 4μ8C and GSK2656157), have been identified (4). Future studies will explore the roles of these small modulators in targeting the UPR and modulating the angiotensin system to understand how each UPR arm is involved in the ANGII/ANG1-7, which can accelerate the discoveries of drugs in treating pulmonary fibrosis.

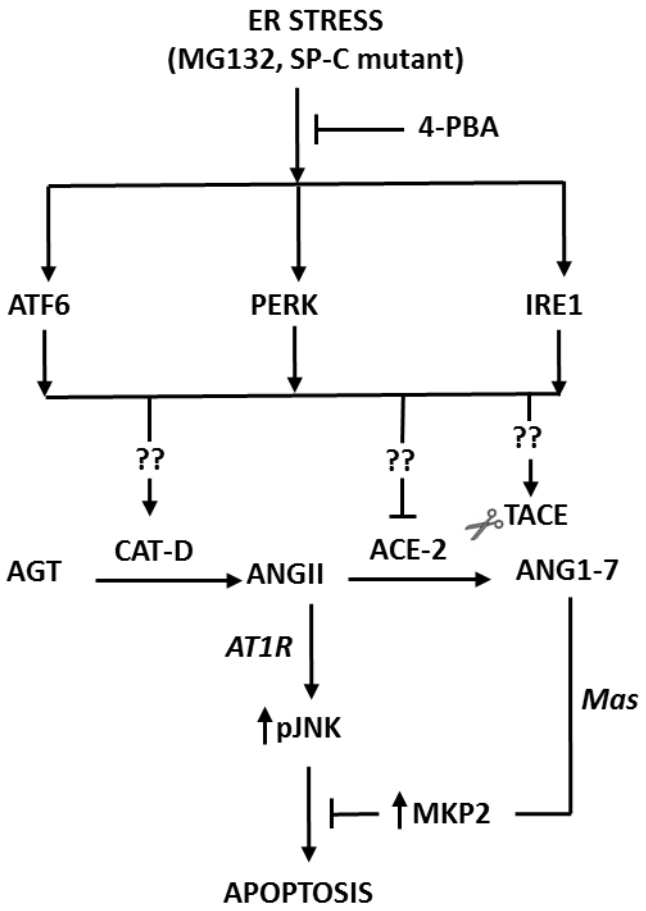


**Figure 5.2. UPR activation regulates ER stress-induced apoptosis of AECs by modulating the autocrine angiotensin system.** ER stress in response to either MG132 or SP-C mutation activates all three UPR arms: the PERK/eIF2α, IRE1/XBP1 and ATF6 pathways. Induction of the UPR upregulates cathepsin D, an aspartyl protease that is required for AEC apoptosis through its production of ANGII from angiotensinogen (10), or downregulates ACE-2 by TACE/ADAM17-mediated ectodomain shedding, each by unknown mechanisms (??) yet to be identified. In turn, cathepsin D activation and ACE-2 downregulation control the ratio of the proapoptotic ANGII and the anti-apoptotic ANG1-7, which inhibits ANGII- induced JNK phosphorylation and apoptosis through *mas*-mediated upregulation of MAP Kinase Phosphatase-2 (MKP-2,1).

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