# ANGIOTENSIN 1-7/MAS PROMOTES ALVEOLAR EPITHELIAL CELL SURVIVAL THROUGH UPREGULATION OF MAP KINASE PHOSPHATASE-2

By

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**ABSTRACT**

**ANGIOTENSIN 1-7/MAS PROMOTES ALVEOLAR EPITHELIAL CELL SURVIVAL THROUGH UPREGULATION OF MAP KINASE PHOSPHATASE-2**

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Apoptosis is now known to be an important regulator of maintaining normal organ homeostasis. However, in recent years experimental studies support the concept that excessive alveolar epithelial cell (AEC) apoptosis contributes to pathogenic conditions in lung. Studies show that local activation of angiotensin system (ANG) in the lung plays a major role in AEC apoptosis. Autocrine generation of angiotensin II (ANGII) an effector peptide, initiates AEC apoptosis through AT1 receptor, phosphorylating c-Jun-N-terminal kinase (pJNK), both of which are required events in AEC apoptosis. Angiotensin converting enzyme-2 (ACE-2) is a vital enzyme that converts ANGII to angiotensin 1-7 (ANG1-7), promoting cell survival by limiting the accumulation of ANGII. Although the downstream signaling mechanisms of ANG1-7/Mas are unclear, experimental studies have shown anti-apoptotic effects of ANG1-7 in AECs.

In this study, the molecular mechanisms by which ANG1-7 and its receptor Mas promote AEC survival are investigated. Previous studies from the Uhal laboratory indicated that under normal conditions ANG1-7 levels are higher than ANGII levels in the AEC culture. Hence, it was theorized that ANG1-7 activates a map kinase phosphatase-2 (MKP-2) and maintains low pJNK levels, as a cell survival mechanism in AECs.

The data show blockade of the Mas receptor diminished the induction of MKP-2 by ANG1-7 which confirmed that Mas acts through MKP-2. Further, silencing MKP-2 abolished the

ability of ANG1-7 to block ANGII-induced phospho-JNK and apoptosis. Silencing of MKP-2 significantly prevented the blockade of all apoptotic markers such as caspase-9, loss of mitochondrial membrane potential (MMP) and DNA fragmentation by ANG1-7. These data support the theory that ANG1-7 upregulates the phosphatase MKP-2 through Mas and thereby maintains low phospho-JNK levels to promote AEC survival.

In conclusion, this study implies that ANG1-7/Mas activation inhibits JNK phosphorylation and apoptosis by constitutively activating MKP-2, and further demonstrates the critical role of the ANG1-7 receptor Mas in AEC survival.

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## KEY TO ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin Converting Enzyme |
| ACE-2 | Angiotensin Converting Enzyme-2 |
| ACEi | Angiotensin Converting Enzyme Inhibitor |
| AEC | Alveolar Epithelial Cell |
| AGT | Angiotensinogen |
| AIF | Apoptosis Inducing Factor |
| ANG1-5 | Angiotensin 1-5 |
| ANG I | Angiotensin I |
| ANG1-7 | Angiotensin 1-7 |
| ANGII | Angiotensin II |
| ANG | Angiotensin System |
| ARB | Angiotensin Receptor Blocker |
| ATI | Angiotensin Type I Receptor |
| BH-3 | Bcl-2 Homology |
| cAMP | Cyclic Adenosine Monophosphate |
| DISC | Death Induced Signaling Complex |
| DUSP | Dual Specific Phosphatase |
| ECM | Extra Cellular Matrix |
| EMT | Epithelial Mesenchymal Transition |
| ER | Endoplasmic Reticulum |

| | |
|---|---|
| ERK | Extra Cellular Regulated Kinase |
| GPCR | G-Protein Coupled Receptor |
| HGF | Hepatocyte Growth Factor |
| IAP | Inhibitor of Apoptosis |
| ILD | Interstitial Lung Disease |
| JNK | Jun N-terminal Kinase |
| KGF | Keratinocyte Growth Factor |
| KIM | Kinase Interacting Motif |
| MAP3K | MAPK Kinase Kinase |
| MAPK | Mitogen Activated Protein Kinase |
| MKP-2 | Map Kinase Phosphatase-2 |
| MLK | Mixed Lineage Kinase |
| MMP | Matrix Metalloproteinase |
| MMP | Mitochondrial Membrane Potential |
| MOMP | Mitochondrial Outer Membrane Potential |
| NEP | Neprilysin |
| NF-κB | Nuclear-factor kappa B |
| PDGF | Platelet Derived Growth Factor |
| PEP | Prolylendopeptidase |
| PKC | Protein Kinase C |
| PLC | Phospholipase C |
| RAS | Renin Angiotensin System |

| | |
|---|---|
| ROS | Reactive Oxygen Species |
| siRNA | Small interfering RNA |
| SP | Surfactant Protein |
| TACE | TNF-α Converting Enzyme |
| TGF | Tumor Growth Factor |
| TNF-α | Tumor Necrosis Factor Alpha |
| VSMC | Vascular Smooth Muscle Cell |

# CHAPTER 1: GENERAL INTRODUCTION

# INTRODUCTION

## Definition of Apoptosis

In recent years, excessive apoptosis of alveolar epithelial cells (AECs) has shown to contribute to progressive lung diseases such as idiopathic pulmonary fibrosis (IPF), acute lung injury (ALI) and chronic obstructive pulmonary disease (COPD, Li, 2004; Uhal, 2003). Apoptosis is a form of programmed cell death that has a fundamental role in maintaining homeostasis which is essential for normal organ development. It is an active, highly regulated physiological process that removes unnecessary cells without initiating an immune response. Although apoptosis is beneficial to maintain normal physiological function, excessive cell death has been reported in many disease types. Therefore, investigation of the factors that control apoptosis is essential to gain insights in certain disease states.

## Characteristics of Apoptosis

During apoptosis, eukaryotic cells undergo a series of morphological and biochemical characteristics that can be distinguished from normal cells. Cells that undergo apoptosis are rapidly phagocytosed by macrophages without damaging the adjacent cells. In normal living cells phosphatidylserine (PS) resides in the inner leaflet of the lipid bilayer, and during early apoptosis, PS can be seen on the outer leaflet of the membrane (Mariño and Kroemer, 2013). Apoptotic cells initially retain the integrity of the plasma membrane. However, during the early process of apoptosis, the membrane forms cell blebs which lead to decreased cell size. Extensive blebbing causes decreased cytoplasm, tightly packed organelles and formation of apoptotic bodies (Hotchkiss et al., 2009). Activation of cysteine proteases known as caspases

play a major role in apoptosis which cleave other cellular proteins and activate endogenous DNases leading to DNA fragmentation (Saraste and Pulkki, 2000). During the late apoptotic phase, nuclear fragments are commonly seen and this event is one of the most commonly measured hallmarks of apoptosis (Henson and Tuder, 2008).

## Major Apoptotic Signaling Pathways

### *Extrinsic Apoptotic Signaling Pathway*

The methodical events in cell death are controlled by an apoptotic signaling network, which includes an extrinsic and intrinsic pathway. The extrinsic pathway is activated when cytokines such as Fas ligand (FasL) and tumor necrosis factor (TNF-α) ligands bind to cell-death receptors. These death-receptors belong to the TNF-α receptor family and their cytosolic regions comprise a death domain. Once the death-receptors are bound by their ligands they form trimers. When the ligand binds to its receptor, the receptor is activated, recruiting factors such as Fas-associated death domain (FADD) and pro-caspases 8/10 that form the death inducing signaling complex (DISC, Fattman, 2008). The caspases are major players in initiating a proteolytic cascade. Caspases are generated in the cell as pro-caspases which are inactive precursors that are activated upon cleavage at aspartic acids by other already activated caspases. The initiator caspases, including caspase 8 and 10 are responsible for activating the executioner caspases that can cleave cellular proteins. Autocatalytic activation of initiator caspase-8/10 activate downstream effector caspase-3/7 eliciting ultimate breakdown of the cell (Elmore, 2007). Moreover, cellular FLICE inhibitory protein (cFLIP) has shown to inhibit apoptosis by interacting with the DISC complex.

***Intrinsic Apoptotic Signaling Pathway***

The intrinsic pathway or the mitochondrial pathway of apoptosis is triggered when an apoptotic stimulus disrupts the balance between pro-apoptotic and anti-apoptotic Bcl-2 proteins. Bcl-2 family of proteins contains both pro and anti- apoptotic proteins that regulate cell survival or death. Bcl-2 itself and Bcl-XL are anti-apoptotic proteins while Bax and Bak are pro-apoptotic proteins that increase permeability in mitochondria. Some pro-apoptotic proteins contain only Bcl-2 homology 3 (BH-3) domain which binds to anti-apoptotic proteins and inhibit cell survival (Hotchkiss et al., 2009). Upon receiving the death signal, the cytoplasmic protein Bax translocates to the outer mitochondrial membrane. Oligomerization of Bax and Bak results in mitochondrial outer membrane permeabilization (MOMP), releasing mitochondrial proteins including cytochrome c (cyt c) and apoptosis inducing factor (AIF, Tait and Green, 2010). Cyt c combines and stimulates oligomerization of apoptosis protease activating factor 1 (Apaf-1) creating a large scaffold complex called the apoptosome which recruits pro-caspase-9. Activation of caspase-9 results in activating the proteolytic cascade and the activation of caspase-3 and 7 leading to apoptosis (Green, 2005).

A cross talk between extrinsic and intrinsic path occurs, when caspase-8 cleaves and activates the pro-apoptotic BH-3 protein Bid to a truncated Bid (tBid) which causes MOMP. Inhibitor of apoptosis (IAP) is a family of proteins that are able to bind to pro-caspases and prevent the activation of caspases. X-chromosome linked inhibitor of apoptosis (XIAP) is a key member of the IAP family that inhibits caspase 3, 7 and 9 and prevents cell death. This inhibition of apoptosis by IAPs, is blocked by certain factors including Smac and Omi, which are released from mitochondria that activate the caspases thus promoting apoptosis (Green, 2005).

## ALVEOLAR EPITHELIAL CELLS

### Type I and Type II Cells

The lung epithelium is composed of type I and II epithelial cells. These cells are present in similar numbers but are functionally distinct. Type I cell has an extended cytoplasm that covers most of the surface area in the lung and is also terminally differentiated. Therefore, type I cell is quite susceptible to injury and cannot initiate self-repair. The thin cytoplasm primarily facilitates respiratory gas exchange and minimizes the diffusion distance between alveoli and pulmonary capillaries (Uhal, 1997).

Type II cell is cuboidal and is the stem cell of the lung. Type II cells are located in the corners of the alveoli. In contrast to type I cells, type II cells are resistant to injury (Selman and Pardo, 2006). The multi-functional type II cell is capable of proliferating and differentiating into both type I and II cells (Uhal, 2008). Type II cell synthesizes, stores and secretes the pulmonary surfactant, a heterogeneous mixture of lipids and proteins to reduce surface tension in the lung (Uhal, 2003). Type II cells are capable of synthesizing immune-modulatory proteins that are important for host defense and turnover of extracellular matrix molecules (Figure 1-1). The equilibrium between type I and type II cells depend on the proliferation, differentiation and cell death of type II cells. Due to the high proliferative capacity of type II cells, during an injury type II cells repair the damaged epithelium and maintain barrier functions. Failure to initiate re-epithelialization leads to destruction of the healthy alveolar epithelium (Uhal and Nguyen, 2013). Hence, loss of type II cells is particularly significant.

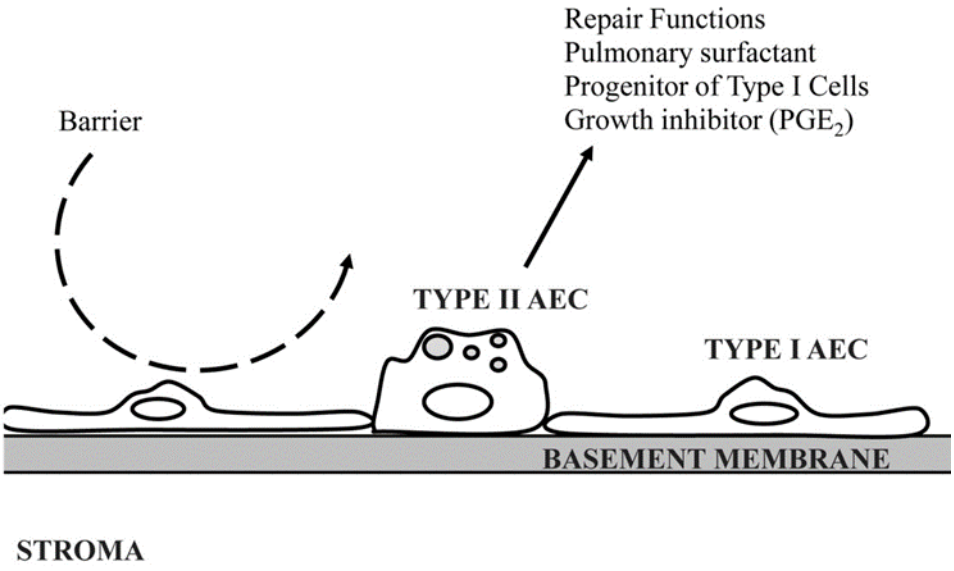


**Figure 1-1: Functions of type I and type II alveolar epithelial cells.**

Type I cells are the squamous cells that facilitate majority of the gas exchange. Type II cells produce the pulmonary surfactant that reduces pulmonary surface tension and is the stem cell of the lung. They also produce factors like prostaglandin (PGE-2) and plasminogen activators that inhibits fibroblast proliferation and degrade fibrin respectively. Type II cells produce matrix-metalloproteinase (MMP) that degrade the extra cellular matrix (ECM).

**Alveolar Cell Defense Mechanisms**

The lung epithelium is equipped with several defense mechanisms to maintain barrier functions. The pulmonary surfactant produced by type II cells, especially surfactant proteins (SPs) A and D bind to pathogens and enable their destruction via alveolar macrophages (Fehrenbach, 2001). Both SP-A and D have been shown to bind to bacteria and viruses and increase phagocytosis. Alveolar macrophages are large mononuclear phagocytes that play a vital role by engulfing various inhaled particles and destroying them. Several studies suggest that alveolar macrophages can release factors that enhance the recruitment of fibroblasts and promote collagen deposition (Uhal et al., 2007). Further, macrophages are known to secrete many enzymes including MMPs, cytokines and growth factors that modulate other cells. These phagocytes interact with a variety of pathogens through cell surface receptors that bind to specific ligands. A recent study demonstrated that angiotensin II (ANGII) upregulates MMP-9 and MMP-2 through partial activation of the ATI receptor in macrophages (Uhal et al., 2007).

Type II cells also produce growth inhibitors like prostaglandin E2 (PGE-2) and matrix metalloproteinases (MMPs) which inhibit fibroblast proliferation and degrade collagen, respectively (Uhal, 2008). Similarly, plasminogen activators convert plasminogen to plasmin that degrades interstitial fibrin and restricts cytokines from reaching the underlying tissue. Toxic agents including industrial dusts, cigarette smoke, air pollutants, and certain disease conditions may weaken or inhibit pulmonary defense mechanisms. Therefore, changes in macrophage function may be central in determining the effectiveness of defense mechanisms.

**Apoptosis in the Lung Epithelium**

Apoptosis in the lung is essential to maintain normal tissue homeostasis. It is vital during development and restoration of the normal lung after injury. The intact lung epithelium protects the underlying tissue by providing a physical barrier. The normal lung cells are generally quiescent; however, when epithelial cells are damaged, it is essential to repair the site of damage in order to maintain the physical barrier (Camelo et al., 2014). Increased alveolar epithelial cell (AEC) apoptosis may lead to a collapse in the barrier and halt its protective functions (Uhal, 2008). Actions that stimulate repeated insults to lung epithelial cells and contribute to dysregulated repair mechanisms are not completely understood (Selman and Pardo, 2006). Further, loss of AECs contributes to a reduction of growth inhibitors and a reduction of matrix metalloproteinase, thus creating a damaged microenvironment (Uhal, 2008). Unlike terminally differentiated type I cells, type II cells are capable of repairing the damaged alveolar epithelium (Li, 2009). Upon injury, damaged type I cells are removed and type II cells begin to proliferate rapidly forming a so called hyperplastic epithelium and then differentiate into type I cells to maintain the integrity of the epithelium (Uhal and Nguyen, 2013). The exact processes of type II cell differentiation into type I cell *in vivo* are not completely understood (Selman and Pardo, 2006). It was shown that type II cell cultures loose lamellar bodies and alter the gene expression exhibiting type I characteristics (Guo et al., 2001b). Type II cells also produce factors that facilitate the migration, differentiation and adhesion of fibroblasts. Thus, injury to type II cells or delayed epithelial repair can result in loss of the intact epithelium allowing the fibroblasts to reach the alveolar air space contributing to the obliteration of the lung architecture.

Alveolar epithelial cell apoptosis is a persistent finding in a number of interstitial lung diseases (ILD). It was demonstrated that upregulation of DNA fragmentation and apoptosis in alveolar epithelial cells, contributed to ILD (Kuwano et al., 1996). Increased expression of pro-apoptotic proteins and decreased expression of anti-apoptotic proteins have been reported in AECs (Uhal, 2008). An earlier study showed increased labeling of fragmented DNA that was used as a marker of apoptosis in lung tissues isolated from experimental animal models treated with bleomycin (Hagimoto et al., 1997).

The Fas receptor is expressed in type II cells and administration of Fas activating antibodies stimulated apoptosis in AECs (Fine et al., 2000). It was also shown that bleomycin, TNF-α and angiotensin II are all physiologically relevant molecules that can induce apoptosis in AECs (Uhal, 2003). The AECs can be exposed to a variety of reactive nitrogen species and under certain conditions $NO_2$ may interfere with epithelial repair and induce apoptosis selectively (Fine et al., 2000). Blockade of AEC apoptosis has shown to reduce lung injury and further, AECs lacking the pro-apoptotic protein Bid, were resistant to tumor growth factor-β (TGF-β) induced cell death (Budinger et al., 2006).

**Lung Epithelial Repair Mechanisms**

The exact signaling mechanisms through which AECs induce a repair process remain unclear, but the current knowledge on the pathway is described in brief. The epithelial repair process consists of epithelial cell-cell communication and epithelial cell-extra cellular matrix (ECM) communication. Inhibition of interleukin-1β (IL-1β) significantly reduced AEC repair *in vitro* suggesting epithelial repair occurs through an IL-1β dependent mechanism (Geiser, 2003).

These data were further strengthened by demonstrating the induction of alveolar epithelial repair by a recombinant form of IL-1β. Evidence shows the involvement of the epidermal growth factor (EGF) and its receptor (EGFR) in alveolar epithelial repair both *in vivo* and *in vitro*. Neutralizing antibodies against tumor growth factor (TGF-α) and EGF, reduced IL-1β induced epithelial repair. Blockade of the EGFR or intracellular signaling pathways inhibited IL-1β induced repair showing through an in vitro model that IL-1β may induce epithelial repair through the EGF/TGF-α pathway (Geiser, 2003).

Hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) are members of the fibroblast growth factor family. These growth factors were shown to protect the epithelium against injury. Several studies done in animal models, suggest a major role of HGF and KGF in epithelial repair after injury. In rats, administration of bleomycin increased the levels of KGF and correlated with AEC proliferation (Barazzone et al., 1999). HGF may also act as a potent mitogen for AECs after injury. Several studies also suggest that effects of KGF on the alveolar epithelium are mediated in part by the EGFR pathway. Treatment with HGF significantly stimulated DNA synthesis and proliferation (Ohmichi et al., 1996). In addition, several studies have shown the anti-apoptotic effects of HGF on alveolar epithelial cells (Uhal, 2008). Further, HGF stimulated proliferation of bronchial epithelial cells were dependent on cyclooxygenase-2 (COX-2) mediated MAPK/Akt pathway (Crosby and Waters, 2010).

**Epithelial to Mesenchymal Transition (EMT)**

EMT is the phenotypic transition of a differentiated epithelial cell to a fully differentiated mesenchymal cell. It is known that, during development or injury to the lung can induce epithelial cells to differentiate to a mesenchymal phenotype to restore the normal architecture (Crosby and Waters, 2010; Willis et al., 2006). But in aberrant would healing models, it is suggested that EMT can contribute to interstitial lung diseases. In the presence of TGF-β alveolar epithelial cells undergo a loss of epithelial markers and express a variety of mesenchymal markers including α-smooth muscle actin (α-SMA), vimentin and type 1 collagen that leads to motility, and cytoskeletal rearrangements. Type II AECs are the main source of surfactant protein- C (SP-C) production and exposure to TGF-β has shown to downregulate SP-C and gain fibroblast like morphology (Willis et al., 2006). E-cadherin is expressed in type II AECs and EMT causes loss of E-cadherin which leads to a reduction in cell–cell communication and contributes to cell migration (Crosby and Waters, 2010).

An earlier study demonstrated exposure of alveolar epithelial cells to TGF-β, expressed morphological changes and fibroblast phenotypic markers (Kasai et al., 2005). Further, siRNA mediated gene silencing of Smad2 inhibited TGF-β induced EMT in AECs. The findings from this study indicated that TGF-β induces EMT in human type II alveolar epithelial cells through Smad2 signaling pathway (Kasai et al., 2005). Similarly, when primary cultures of rat AECs were exposed to TGF-β, the cells increased expression of mesenchymal markers and decreased expression of epithelial markers (Willis et al., 2005). These data were strongly supported by another study conducted in mice expressing β-galactosidase (β-gal) that allows to determine whether EMT occurs *in vivo*. Overexpression of TGF-β caused accumulation of β-gal positive

cells expressing mesenchymal markers (Kim et al., 2006). Nevertheless, complete understanding of the signaling process in EMT and its dysregulation is needed.

## RENIN ANGIOTENSIN SYSTEM (RAS)

The renin-angiotensin system (RAS) plays a central role in regulating blood pressure. Renin is produced in the juxtaglomerular cells and a decrease in blood pressure or increased activity in the sympathetic nervous system can release this hormone to the circulatory system. The circulating renin can cleave liver produced angiotensinogen (AGT) to generate angiotensin I (ANG I). Angiotensin converting enzyme (ACE) hydrolyzes ANG I to angiotensin II (ANGII). Binding of ANGII to ATI exerts harmful actions, if not properly counterbalanced. ANGII narrows blood vessels (vasoconstriction) increasing blood pressure. ANGII releases vasopressin and aldosterone which act on the distal tubule, mediating water and sodium retention (Fyhrquist and Saijonmaa, 2008; Simões E Silva and Flynn, 2012). The effects of the ATII receptors have not been completely understood but generally are known to counteract ANGII induced signaling (Robles et al., 2014). In the last decade, several angiotensin peptides have been discovered including angiotensin III (ANGIII), angiotensin IV (ANG IV), and angiotensin I-7 (ANG1-7, Robles et al., 2014).

Recently discovered, prorenin/renin receptor increases the conversion of AGT to ANG I and there by contributes to accumulation of ANGII when the receptor is bound by renin. Also this interaction stimulates intracellular signaling and enhances synthesis of tumor growth factor (TGF-β) signaling, collagen and fibronectin in the kidney (Nguyen, 2011; Robles et al., 2014). Recent discoveries show that classical RAS is expressed locally within tissues, independent or

dependent of the circulatory RAS. In the lung a local RAS is expressed independent of the circulatory RAS and is known as the ANG system. Numerous experimental studies with both animal models and human tissues ex vivo now support the concept that activation of a local angiotensin (ANG) system plays a major role in lung injury**.** A subset of these studies demonstrated the existence of an intrinsic (i.e., all components expressed by the target cell itself) angiotensin system in lung alveolar epithelial cells.

**Local Angiotensin (ANG) System**

Apoptotic causing agents induce apoptosis in AECs by upregulation of AGT synthesis (Figure 1-2). AGT is a 58 kDa protein which is the only known precursor for angiotensin peptides (Uhal, 2003). In response to Fas and TNF-α, AGT synthesis was increased in human alveolar epithelial-derived cell line A549 and moreover, antisense oligonucleotides against AGT completely abrogated apoptosis (Wang et al., 1999, 2000a). Those data clearly show the autocrine production of AGT locally in AECs. Cleavage of the NH2-terminal end of AGT by cathepsin D (cat-D) or other aspartyl proteases generates ANG I which is the substrate of angiotensin converting enzyme (ACE, Li et al., 2004) The biologically active peptide angiotensin II (ANGII) is generated from cleavage of two amino acids from the COOH end of ANG I. ACE is the primary enzyme that could generate ANGII. However, chymase or other peptidases can form ANGII, an octa-peptide that has shown detrimental effects in many cell types including AECs (Uhal et al., 2012). In lung epithelial cells, ANGII induced apoptosis could be blocked by ATI receptor antagonist losartan but not by an ATII antagonist conforming that the physiological receptor for ANGII in AECs is ATI (Papp et al., 2002). Angiotensin converting enzyme -2 (ACE-2)

is an vital enzyme that removes a single amino acid from the COOH end of ANGII, to generate angiotensin 1-7 (ANG1-7, Uhal et al., 2011). The hepta-peptide ANG1-7 has shown to inhibit many injurious actions of ANGII through its receptor Mas which belongs to the G-protein coupled receptor (GPCR) family (Uhal et al., 2012). Inhibition of ACE-2 by a competitive inhibitor DX-600 or siRNA meditated knockdown, caused a significant increase in ANGII levels (Uhal et al., 2011). Therefore ACE-2 demonstrates a protective role against AEC apoptosis by limiting the accumulation of ANGII. In AECs, a balance between ANGII and ANG1-7 is a critical determinant of cell death and survival.

***Angiotensin Receptors and Signaling***

ANGII is a vasoconstrictor and has demonstrated pro-apoptotic, proliferative, hypertrophic, fibrotic, mitogenic and tissue remodeling actions. There are two types of ANGII receptors; ATI and ATII, both of which are members of the seven-transmembrane domain, GPCR superfamily but are functionally distinct with a sequence homology of 30% (Goodfriend et al., 1996). Two types of ATI receptors have been identified; ATI A and ATI B which are present in rodents but not in humans (Guo et al., 2001a). The ANGII/ATI receptor signaling pathway is mediated by heterotrimeric $G_{q/11}$-proteins that activate phospholipase C (PLC, Goodfriend et al., 1996). Cleavage of Phosphatidylinositol 4, 5-bisphosphate (PIP2) by PLC yields inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG). DAG activates Protein kinase C (PKC) which can add a phospho group to serine/threonine in cellular proteins and stimulate proliferation. In AECs, both ATI and ATII mRNA are present but were demonstrated that ATI is the main receptor responsible for JNK phosphorylation and AEC apoptosis. Signal transduction of ATII receptor is

still unclear although, in several cell types ATII receptor has shown to activate a tyrosine phosphatase and counteract ANGII induced effect through ATI receptor (Papp et al., 2002).

***Angiotensin Converting Enzymes (ACE and ACE-2)***

The balance between ACE and ACE-2 plays a major role in many diseases and ACE-2 is known to counteract actions of ACE. Both of these enzymes are important regulators of the ANG system. ACE is a type 1 transmembrane protein and two forms of ACE has been reported; somatic ACE and germinal ACE (Danilczyk et al., 2003). Somatic ACE is composed of two active-site domains; N-domain and C-domain both of which differ in substrate specificity whereas germinal ACE has a single active site (Danilczyk et al., 2003). ACE converts the decapeptide ANG I to ANGII and also degrades the vasodilator bradykinin. Somatic ACE can be cleaved by a secretase from the cell surface although the biological importance of soluble ACE is poorly understood (Tipnis et al., 2000). In AECs, blockade of ACE by ACE inhibitors such as lisinopril has shown to prevent apoptosis (Filippatos and Uhal, 2003). ACE-2, a 805 amino acid, type 1 transmembrane glycoprotein, was discovered recently and is a close homologue of ACE. Unlike ACE, ACE-2 contains a single active-site domain which has a high substrate specificity of ANGII. ACE-2 also hydrolyzes ANG I to generate ANG 1-9, but relatively poorly. The large extracellular active site of the ACE-2 shows 40% overall sequence identity with the N-domain and C-domain of ACE (Zisman, 2005). ACE-2 can be released from the cell membrane by ADAM 17 which belongs to the adamalysin protein family. Elevated levels of sheded ACE-2 has been reported in myocardial dysfunction (Clarke and Turner, 2011). Knockdown or competitive inhibition of ACE-2 has shown to alter the levels of ANGII and ANG1-7 (Uhal et al., 2011). Further, over-

expression of ACE-2 has demonstrated to treat several diseases in experimental models and has a protective role against AEC apoptosis (Imai et al., 2010).

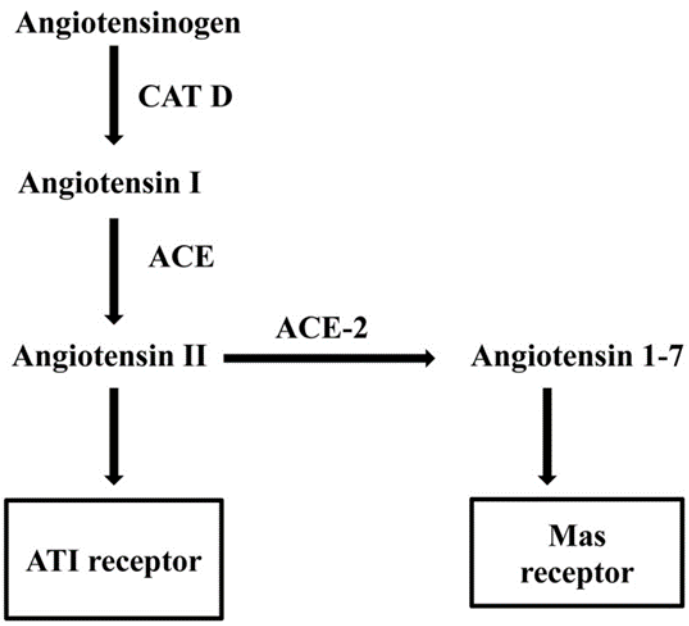


**Figure 1-2: Local angiotensin system in alveolar epithelial cells.**

Apoptotic inducers activate angiotensinogen which is cleaved by cathepsin D (cat D) to generate angiotensin I (ANG I). Angiotensin converting enzyme (ACE) generates angiotensin II by cleaving two amino acids from ANG I. Angiotensin converting enzyme-2 (ACE-2) cleaves a single amino acid from ANGII to generate angiotensin 1-7, the ligand for Mas receptor.

## ANG SYSTEM AND AEC APOPTOSIS

Recently, experimental studies have demonstrated the involvement of ANG system in AEC apoptosis. In response to apoptotic inducers such as Fas ligand, TNF-α and bleomycin, AGT is produced in cultured AECs which after enzymatic cleavage generates ANGII (Li et al., 2003a; Wang et al., 1999, 2000a). Antisense oligonucleotides against AGT mRNA, neutralizing antibodies against ANGII, ACE inhibitors (ACEi) and ATI receptor antagonists have all blocked apoptosis in AECs (Uhal et al., 2012). Collectively, these studies demonstrated the local production of ANGII and activation of ATI receptor. In primary cultures of rat alveolar epithelial cells, ANGII induced apoptosis measured by nuclear fragmentation was blocked by chelerythrine, an inhibitor of PKC which suggests the activation of PKC downstream of ANGII/ATI (Papp et al., 2002). Phosphorylation of jun-N-terminal kinase (pJNK) is a required event in AEC apoptosis (Figure 1-3). ANGII induced pJNK was blocked by ATI receptor antagonist but not by an ATII receptor antagonist, showing that ATI receptor mediates the induction of pJNK (Uhal et al., 2011). Clearly, these data suggest ATI is the functional receptor that mediates JNK phosphorylation and apoptosis in AECs. Activation of caspase-9 by ANGII shows that AEC apoptosis occurs through the mitochondrial pathway. Further, this activation of caspase-9 was prevented by a JNK inhibitor. Blockade of ACE-2, one of the beneficial enzymes that generate anti-apoptotic peptide ANG1-7, by DX-600, a competitive inhibitor induces caspase-9 in AECs (Uhal et al., 2011). Both ANGII and bleomycin induced pJNK was blocked by pre-incubating the cells with ANG1-7. This blockade was prevented by A779, the receptor antagonist for Mas, showing the importance and presence of the Mas receptor in AECs. Similar results were obtained with propidium iodide (PI) assay that measures nuclear fragmentation.

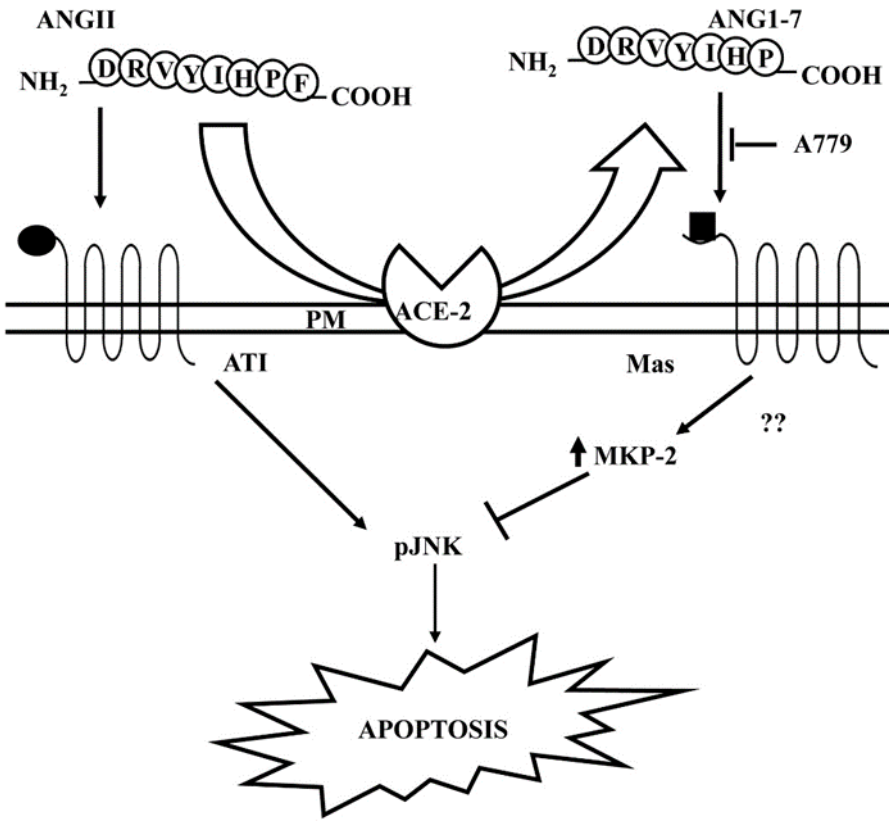


**Figure 1-3: Known signaling mechanisms in alveolar epithelial cells (AECs).**

The octapeptide ANGII induces JNK phosphorylation and apoptosis through the AT1 receptor (Uhal et al., 2011). ACE-2 degrades the pro-apoptotic ANGII to the anti-apoptotic ANG1-7, which inhibits both JNK phosphorylation and apoptosis through the Mas receptor. These inhibitory effects of ANG1-7 are blocked by A779, a specific antagonist of Mas. Current studies suggest that ANG1-7/Mas activation prevents JNK phosphorylation by constitutively activating the JNK-selective map kinase phosphatase-2 (MKP-2) and further, demonstrate the involvement of the Mas receptor in MKP-2 activation. The mechanism(s) by which Mas activation induces MKP-2 (??) are currently unclear (PM ¬– plasma membrane).

**Blockade of Apoptosis in AECs**

Since apoptosis requires activation of many signaling proteins, in the recent years studies have shown blockade of apoptosis holds a great pharmacological potential to prevent lung diseases that involve apoptosis. In a rat model, it was demonstrated excessive collagen deposition after administration of bleomycin was blocked by captopril , an angiotensin converting enzyme inhibitor (ACEi) or by a broad spectrum caspase inhibitor (ZVADfmk) that inhibits the activity of caspases (Filippatos and Uhal, 2003). Deletion of genes that are necessary for apoptosis have proven to be beneficial (Uhal et al., 2012). This was shown by mice lacking Bid, one of the pro-apoptotic proteins demonstrated resistance to transforming growth factor (TGF-β1) induced cell death in alveolar epithelial cells (Budinger et al., 2006). Induction of AEC apoptosis by fas ligand, TNF-α or bleomycin was abrogated by ACEi's or saralasin (non-selective ATI antagonist). Apoptosis was also blocked by antisense oligonucleotides against AGT or by antibodies that neutralize ANGII. Collectively, these studies demonstrated the autocrine production of ANGII regardless of the apoptotic stimuli (Filippatos and Uhal, 2003; Li et al., 2003a; Wang et al., 1999, 2000a). It was demonstrated that ANGII induced apoptosis in A549 cells or in primary rat cells were inhibited by ATI selective blocker losartan but not by ATII selective blocker PD-126055 (Papp et al., 2002).

**ANG System in Heart**

The contribution of ANGII as an apoptotic regulator was first hypothesized by cardiologists. The local presence and synthesis of the ANG peptides in the heart suggest the modulation of cardiac structure and function by ANG peptides (Filippatos et al., 2001). Clinical

and experimental studies have documented the vital role of ANGII in pathological conditions of the heart. ANGII has been shown to upregulate AGT mRNA synthesis, activation of p53 and JAK/STAT pathway in cardiac myocytes (Dostal and Baker, 1999). ANGII induced apoptosis in neonatal cardiac myocytes were blocked by ATI selective antagonists (Filippatos and Uhal, 2003). Further ANGII induced cardiac damage in Sprague-Dawley rats were blocked by ACE-2 gene transfection using lenti-viral vectors (Santos et al., 2013). The hepta-peptide ANG1-7 has shown to attenuate cardiac remodeling. This was further investigated by Tallant et al. in cardiac fibroblasts (McCollum et al., 2012a). ANGII stimulated phosphorylation of extracellular signal regulated kinase (ERK1/ERK2) was blocked by ANG1-7 through upregulation of dual specific phosphatase-1 and the modulatory effects of ANG1-7 were blocked by Mas receptor antagonist. These data show the involvement of the ANG system in heart and beneficial effects of the ANG1-7/Mas pathway.

**ANG System in Kidney**

Proliferation of resident renal cells and deposition of extra-cellular matrix components are well-studied features of progressive renal diseases. A significant amount of studies have demonstrated the involvement of a tissue specific ANG system in pathological conditions in the kidney. ACE inhibitors and ATI selective antagonists have demonstrated beneficial actions against ANGII induced apoptosis and cell proliferation in kidney disease (Filippatos and Uhal, 2003). Experimental studies have demonstrated a protective role of ACE-2 in different models of renal damage by genetic deletion of ACE-2 that aggravates the pathological conditions (Santos et al., 2013). Along similar lines, studies have reported a downregulation of ACE-2

associated with kidney disease and moreover, ANGII induced oxidative stress was attenuated by a recombinant form of ACE-2 (Simões E Silva and Flynn, 2012). Although, the exact ANG1-7 signaling is unclear and ANG1-7 effects can be quite complex in the kidney, studies have reported the inhibition of ATI receptor mediated signaling by ANG1-7 in nephron injury (Brewster and Perazella, 2004). It is also documented that ANG1-7 effects could be controversial in the kidney and this might be due to cell type specificity and differences in models that are used (Santos et al., 2013).

**ANG System in Vascular Smooth Muscle Cells (VSMCs)**

Angiotensin peptides play a key role in regulating vascular reactivity and experimental studies have suggested a vital role of ANGII in VSMCs. ANGII has been found to exert hypertrophy and in some cases hyperplasia. Stimulation of ANGII/ATI pathway activates PLC and intracellular $Ca^{2+}$, in addition to the activation of NADH oxidase in VSMCs (Griendling et al., 1997). Administration of ANGII significantly stimulated proliferation in VSMCs isolated from aorta of Sprague-Dawley rats measured by DNA synthesis (Freeman et al., 1996; Tallant et al., 1999). The proliferation of cells was attenuated after incubation with ANG1-7 showing the regulation of vascular growth by the two major peptides in the ANG system. Further, ANGII induced activity of ERK1/ERK2 was significantly prevented by ANG1-7 (Tallant and Clark, 2003). The signaling mechanisms of ANG1-7 in VSMCs have been investigated and experimental data illustrate that prostacyclin, nitric oxide (NO) and prostaglandin have shown to inhibit VSMC growth. Further, this inhibition occurs through a cyclic adenosine monophosphate (cAMP)

mediated pathway which was demonstrated through pharmacological agents that induce cAMP levels can contribute to inhibition of VSMC proliferation (Tallant et al., 1999).

## ANGIOTENSIN 1-7 (ANG1-7)/ MAS RECEPTOR

The local ANG system has recently been extended with new key players that oppose actions of ACE/ANGII/ATI. The components of the new axis consist of ACE-2/ANG1-7/Mas that have shown to exert many beneficial actions in many cell types including in AECs.

The biologically active peptide ANG1-7 is one of the key regulators in the locally expressed ANG system in AECs. ACE-2 degrades the pro-apoptotic, proliferative and vasoconstrictive octa-peptide ANGII to generate anti-apoptotic, anti-proliferative and vasodilatory hepta-peptide ANG1-7. Recent data demonstrated the importance of ANG1-7 which counteracts ANGII induced deleterious effects in many tissues including in the lung, heart, vascular smooth muscle cells and kidney. The hepta-peptide was first discovered in the brainstem as a product of ANG I and little over 20 years since its discovery, a large body of evidence has shown the importance of ANG1-7, especially after the discovery of its receptor Mas.

It was found that the inhibitory effects of ANG1-7 was blocked by the receptor antagonist A779 (Asp-Arg-Val-Tyr-Ile-His-D-Ala) which was discovered by Santos et al (Santos et al., 1994). This synthetic analogue of ANG1-7 showed to inhibit anti-diuresis in water loaded rats and was unable to block any of ANGII induced agonist effects. Moreover, A779 (D-Ala-ANG1-7) did not displace iodine labelled ANGII in tissues that are rich of ATI receptor subtype. Further, changes in blood pressure by ANG1-7 were blocked by A779 but not by an ATI or ATII

antagonist. Collectively, the data demonstrate that A779 is a selective antagonist for ANG1-7 (Santos et al., 1994).

Before the discovery of Mas, Rent et al. showed vasodilation in rabbit afferent arteriole by ANG1-7 was not blocked by ATI or ATII selective antagonists but was blocked by A779 compound (Ren et al., 2002). Similarly, vasodilation of microvessels in the mesenteric circulation was not blocked by losartan but by A779 (Oliveira et al., 1999). These data were strengthened by similar studies done in other cell types. VSMC growth by ANGII was opposed by the effects of ANG1-7. The blockade was not attenuated by ATI or ATII selective antagonists but by A779 (Freeman et al., 1996). These data provided the evidence of a novel ANG1-7 specific receptor. However, at higher concentrations of ANG1-7, studies have shown that it can produce ANGII like effects through binding to ATI (Santos et al., 2000).

The Mas proto-oncogene was discovered by its tumorigenic properties that encodes a seven transmembrane receptor which was considered as an orphan GPCR (Santos et al., 2003). The tumorigenic properties of Mas appears to be really low and it was initially thought to be the functional receptor for ANGII (Jackson et al., 1988). Santos et al. demonstrated through radio-ligand binding studies in mouse kidney, that the physiological receptor for ANG1-7 is the G-protein coupled receptor Mas (Santos et al., 2003). In this study the authors demonstrated the binding of $I^{125}$ labelled ANG1-7 with high affinity to Mas transfected CHO cells. Moreover, this binding was displaced by both unlabeled ANG1-7 and A779 with high affinity. To examine the functional effect of ANG1-7, the release of arachidonic acid (AA) was measured in CHO cell transfected with the Mas receptor. The induction of AA release was blocked by A779 but not ATI or ATII selective antagonists, demonstrating a specific receptor for ANG1-7 distinct from ATI

or ATII (Santos et al., 2003). Additionally, relaxation of the aortic rings in rats was investigated with ANG1-7 treatment. Mas deficient aortas lost their ability to induce relaxation effect in response to ANG1-7 compared to the wild type demonstrating Mas as a functional receptor for ANG1-7 (Santos et al., 2003).

It is now evident that GPCRs may exist as homo or hetero dimers. Recently, it was discovered that ANG1-7/Mas mediated inhibition occurs through interaction with ATI receptor. In transfected mammalian cells ANGII/ATI induced generation of inositol phosphates and intracellular $Ca^{2+}$ were reduced by half after the expression of Mas receptor. The authors also demonstrated the formation of a hetero-oligomeric complex between ATI and Mas receptor that does not respond to agonists or antagonists of the both receptors (Kostenis et al., 2005).

Along similar lines, a different group demonstrated that ATII receptor is an antagonist of ATI receptor that prevents ANGII signaling in fetal fibroblasts. ATI receptor induced inositol phosphate levels were augmented in fibroblasts after a reduction of ATI/ATII hetero-dimerization when antisense construct against ATII was used. Further, ANGII induced signaling was blocked by ATI selective antagonist but not by ATII antagonist. But the reduction in ATI/ATII hetero-dimer complex caused an induction in ATI signaling demonstrating that ATI signaling is prevented (at least partially) by the ATII receptor, forming a hetero-dimerization between the two receptors (AbdAlla et al., 2001). Castro et al. showed, ANG1-7 mediated vasodilation was significantly influenced in the presence of losartan demonstrating a complex interaction between ATI/Mas and possibly with ATII receptors (Castro et al., 2005). However, more studies are required to elucidate the functional interactions among these receptors.

**ANG1-7 Synthesis and Catabolism**

The physiological and the most accepted pathway of ANG1-7 generation is hydrolysis of ANGII by ACE-2 (Figure 1-4). The enzyme ACE-2 can also generate ANG I-9 by ANG I. However, It is known that ACE-2 has 400 fold higher affinity for ANGII than ANG I (Xu et al., 2011). ACE or Neprilysin (NEP) cleaves two amino acids from ANG 1-9 to generate ANG1-7. NEP, thimet oligopeptidase and prolylendopeptidase (PEP) can remove three amino acids directly from ANG I to generate ANG1-7.

The hydrolysis of ANG1-7 has been investigated in the rat lung. The authors demonstrated the breakdown of radio-labeled ANG1-7 primarily to ANG1-5. ACE has been shown to bind to ANG1-7 with high affinity and inhibition of ACE by lisinopril abrogated the generation of ANG1-5. Further, degradation of ANG1-5 yielded ANG3-5, independent of ACE and might due to other enzymes like diamino-peptidases (Allred et al., 2000).

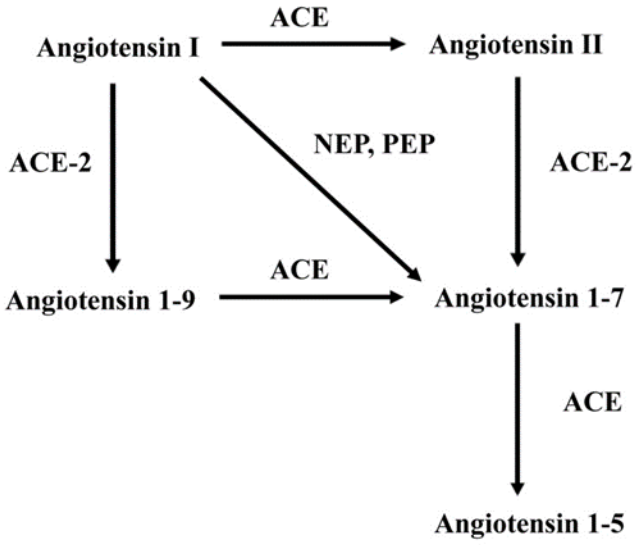


**Figure 1-4: Angiotensin 1-7 synthesis and catabolism.**

ANG1-7 can be synthesized by several different enzymatic mechanisms (Allred et al., 2000). ACE-2 degrades the octa-peptide ANGII to the hepta-peptide ANG1-7. ANG I can be metabolized by neprilysin (NEP), prolylendopeptidase (PEP) into ANG1-7. ACE-2 also can cleave ANG I to generate angiotensin 1-9 (ANG 1-9) which then is degraded by ACE resulting in ANG1-7. ACE is also involved in degrading ANG1-7 to angiotensin 1-5.

**Inhibitory Actions of the ANG1-7/Mas Pathway on Pulmonary Injury**

Exactly how the ANG1-7/Mas axis affects injurious signaling pathways is currently a topic of intense focus. The protective actions of the ANG1-7/Mas axis on lung cells in non-neoplastic lung injury are briefly discussed here. The local ANG system is activated after tissue injury in a variety of organs to promote repair, but abnormalities in the process promote lung injury. Many experimental studies have elucidated the contribution of AEC apoptosis to the pathogenesis of lung fibrosis (Fattman, 2008; Hagimoto et al., 1997; Li et al., 2006). Many years ago and more recently, seminal research works reported data to support the concept that the death of AECs, by itself, could create a profibrotic microenvironment without the involvement of an inflammatory response (Uhal and Nguyen, 2013). Consistent with this concept, blockade of apoptosis of AECs during lung injury by angiotensin receptor blockers (ARBs), ACEi or by a broad-spectrum caspase inhibitor decreased the fibrotic response in animal models (Filippatos and Uhal, 2003). However, issues that might limit the applicability of this approach to human subjects have been discussed, such as potential side effects, gender differences and in the case of ARBs, the potential for systemic hypotension in some patients (Ferreira et al., 2012). Although ACEi have shown to reduce lung fibrogenesis in some animal models, clinical trials of ACE inhibitors in humans have failed to detect beneficial effects on lung fibrosis. This might be explained by the presence of other enzymes independent of ACE that could generate ANGII. Thus, it is crucial to understand the underlying mechanisms of the counter-regulatory axis ACE-2/ANG1-7/Mas, which may hold potential for future therapeutics for lung diseases.

In AECs, constitutively expressed ACE-2 converts the pro-apoptotic octapeptide ANGII to the anti-apoptotic heptapeptide ANG1-7, and thereby limits the accumulation of ANGII to

promote cell survival (Uhal et al., 2012). Evidence for a beneficial role of ACE-2/ANG1-7 is strengthened by *in vivo* studies of experimental animals that used genetic manipulation of ACE-2 or specific inhibitors of ACE-2 to establish a protective role of the enzyme (Soler et al., 2008). Previous work in this laboratory showed that ACE-2 is protective against experimental fibrosis, but is down-regulated in both human lung fibrosis and experimental lung fibrosis in animal models (Li et al., 2008). Uhal et al. demonstrated that ACE-2 mRNA, protein and enzymatic activity were severely decreased in lung biopsy specimens isolated from IPF patients and also in the lung tissue of experimental animals made fibrotic by administration of bleomycin (Li et al., 2008). In these studies, intratracheal administration of either ACE-2-specific siRNAs or DX600, a competitive inhibitor of ACE-2, enhanced bleomycin-induced lung collagen accumulation. Moreover, in the lungs of animals in which ACE-2 was manipulated in these ways, ANGII levels were increased and the resulting increase in lung collagen was blocked by an ANG receptor blocker. Together, these studies showed that ACE-2 is protective against lung fibrogenesis by controlling local ANGII generation.

In the lungs of patients with IPF, many alveolar epithelial cells are proliferating in the so-called "hyperplastic epithelium" described by pathologists, whereas AECs in the normal lung are primarily quiescent (Li, 2004). On this basis, it was hypothesized that cell cycle regulation plays an important role in ACE-2 expression by AECs. This was verified in a recent study that showed significant differences in ACE-2 mRNA, protein and enzymatic activity in sub-confluent (proliferating) vs. post-confluent (quiescent) human lung cells in culture, and within normal or fibrotic human lung specimens (Uhal et al., 2013a). The data clearly showed a down-regulation of ACE-2 mRNA, protein and enzymatic activity in proliferating cells and an up-regulation in

quiescent cells. Additionally, the up-regulation of ACE-2 that occurs in cells approaching density-dependent quiescence *in vitro* is blocked by the transcription blocker actinomycin D or by an inhibitor of JNK phosphorylation. Taken together, these results illustrated the cell cycle-dependent and JNK-mediated regulation of ACE-2 expression in AECs.

Recent work in our laboratory showed that both ANGII generation and JNK phosphorylation are required events in AEC apoptosis and subsequent lung injury (Uhal et al., 2011). It was speculated that ACE-2, as well as its product ANG1-7, might regulate AEC apoptosis. This theory was confirmed by the findings that ANG1-7 could block JNK phosphorylation, caspase activation and nuclear fragmentation in a cultured mouse lung epithelial cell line (MLE-12 cells) or in primary cultures of rat lung alveolar type II epithelial cells (Uhal et al., 2011). Furthermore, pretreatment with A779, a specific antagonist of the Mas receptor, prevented the inhibitory actions of ANG1-7 and thus implicated the involvement of Mas receptor (Uhal et al., 2011). A subsequent study of the human lung epithelial cell line A549 and primary cultures of human lung AECs evaluated apoptosis of these cells, induced by either MG132 (a proteasome inhibitor and inducer of ER stress) or by the surfactant protein C (SPC) BRICHOS domain mutation G100S (an inducer of the Unfolded Protein Response and ER stress), one of several recently discovered SPC mutations that cause interstitial lung disease. In response to either of these inducers, the apoptosis was completely abrogated by ANG1-7 (Uhal et al., 2013b). Specifically, ANG1-7 prevented the induction of caspases, loss of mitochondrial membrane potential, cytochrome c release, JNK phosphorylation and nuclear fragmentation in the cultured human AECs. Further, the Mas antagonist A779 blocked the inhibition of apoptosis by ANG1-7 and demonstrated the involvement of Mas. This study also demonstrated a

reduction of ACE-2 expression when the cultured AECs were challenged with the proteasome inhibitor MG132 or the SPC mutant G100S. This reduction was prevented by an inhibitor of the ACE-2 ectodomain shedding enzyme ADAM17/TACE (TAPI-2). Together, these data demonstrate that AEC apoptosis is mediated by the autocrine ANGII/ANG1-7 system expressed by these cells, and suggest that the hepta-peptide ANG1-7 may hold therapeutic potential for lung diseases in which the UPR and/or ER stress play a role in pathogenesis. The exact mechanisms of the activation of the ANG system in response to ER stress are currently unclear but are under investigation.

Consistent with these observations, Shenoy et al. demonstrated that intratracheal administration of a lentiviral-packaged ANG1-7 expression construct or ACE-2 cDNA into Sprague Dawley (SD) rats significantly inhibited bleomycin-induced collagen deposition, expression of TGF-β mRNA and AT1 receptor protein levels in the rat lungs (Shenoy et al., 2010). Additionally, protective effects against lung fibrosis were also obtained by overexpression of ACE-2. This study is consistent with a previously published study that demonstrated that exposure of cultured rat or human AECs to bleomycin *in vitro* caused a robust expression of AGT mRNA and the processed peptide ANGII, both of which are required for the apoptotic response of these cells (Li et al., 2003a). Attempts to determine which profibrotic genes might be activated in response to ANGII have shown the induction of TGF-β and α-collagen-1 mRNA levels *in vitro* in human fetal lung (HFL-1) cells exposed to the octapeptide (Meng et al., 2013a). Pre- or co-incubation with ANG1-7 prior to the application of ANGII inhibited the induction of the profibrotic genes. However in this study, pre-incubation with A779 did not prevent the inhibitory actions of ANG1-7. The cause for this incongruity is not

clear, but may depend on the cell type specificity (fibroblast vs. epithelial cell) or the experimental conditions that were used (Filippatos and Uhal, 2003).

Acute respiratory distress syndrome (ARDS) is one of the most devastating forms of acute lung injury (ALI). Each year in the United States around 200,000 patients suffer from ARDS (Wösten-van Asperen et al., 2011). Apoptosis of AECs has been discovered in the lungs of ARDS patients and was associated with increased Fas/FasL expression (Albertine et al., 2002). It is currently believed that anomalies of the ANG system contribute to the pathogenesis of ARDS. About 60% of ARDS patients are shown to develop pulmonary fibrosis with increased mortality rates (Phua et al., 2009). A considerable number of *in vivo* studies demonstrate the beneficial actions of the ACE-2/ANG1-7 axis in acute lung injury in several animal models. For example, intratracheal administration of lipopolysaccharide (LPS) induced acute lung injury in C57BL/6 mice, which resulted in substantial induction of collagen accumulation, pulmonary edema and inflammation (Chen et al., 2013b). However, subcutaneous infusion of ANG1-7 significantly reduced hydroxyproline levels (a marker of total collagen) as well as TGF-β1 and Smad2/3 protein levels. Treatment with A779 prevented the protective effect of ANG1-7 on collagen deposition and lung remodeling, observations that provide *in vivo* evidence that Mas mediates the protective role of ANG1-7 on lung injury and fibrogenesis (Figure 1-5).

Similarly, overexpression of a recombinant form of ACE-2 prevented ALI induced by acid aspiration or sepsis in mice (Imai et al., 2005). The same authors also demonstrated that mice deficient in ACE had markedly decreased ALI. On the other hand, the importance of ACE-2 is strengthened by the use of ACE-2 knockout mice (Hamming et al., 2007). Experimental ARDS induced in mice by acid aspiration was more severe in ACE-2 knockouts compared to wildtype

controls that express functional ACE-2; the loss of ACE-2 in the knockout mice increased neutrophil accumulation and worsened pulmonary edema. These studies demonstrated the protective role of ACE-2 *in vivo* in models of ALI and showed that part of this defensive role is due to limitation of the accumulation of ANGII. Recently, many studies have accumulated to support the view that an imbalance between the enzymatic activity of ACE and ACE-2 determines the local tissue levels of ANGII and ANG1-7. For example, a study conducted to determine pulmonary ACE and ACE-2 activity in patients with ARDS, demonstrated increased ACE activity and decreased ACE-2 activity compared to the control group (Wösten-van Asperen et al., 2013). In an animal model of ARDS, the reduction of ACE-2 activity was also present, but could be reestablished by *in vivo* treatment with ANG1-7. These findings are promising but since the clinical study was limited to fourteen ARDS patients, larger clinical studies are needed for confirmation. Along the same line of thinking, Imai et al*.* demonstrated that pharmacological inhibition of AT1 receptor or ACE-knockout mice showed improved ALI symptoms in the absence of functional AT1 receptor or ACE (Imai et al., 2008). On the basis of this work and that summarized in preceding paragraphs, it has been theorized that ACE/ANGII/AT1 can promote ALI, but the counter-regulatory axis ACE-2/ANG1-7 is protective against ALI.

Related experimental studies showed that ALI in mice following hindlimb ischemia-reperfusion (LIR) is also due to the dysregulation of the ANG system (Chen et al., 2013a). Changes in the ACE/ACE-2 mRNA level and protein levels were measured after 2 hour of hindlimb ischemia in mice. In addition, ANGII and ANG1-7 levels in the blood serum and in lung tissues were measured by enzyme-linked immunosorbent assay. In the beginning of the

reperfusion period, the authors found higher levels of ANG1-7 than ANGII, but in later stages of reperfusion ANGII levels were higher than ANG1-7 levels. This change agreed with varying levels of ACE/ACE-2 expression (Chen et al., 2013a). Consistent with other works mentioned above, genetic deletion of ACE-2 showed increased disease progression in this model. Collectively, the above studies all demonstrate the protective role of ACE-2 in lung injury.

On the other hand, the efficacy of ANG1-7 administration *in vivo* has been less well documented to date, in part due to the many proteases that degrade the heptapeptide very rapidly. This problem was bypassed by the addition of a thioether ring to the peptide to form cyclic ANG1-7 (cANG1-7), which has been shown to increase resistance to proteolytic degradation *in vivo* in Sprague Dawley rats (Kluskens et al., 2009). Experimental studies have found enhanced stability of the cANG1-7 and also evidence that it binds to the ANG1-7 receptor Mas with high affinity. Efficacy of the cyclic analog of ANG1-7 was confirmed by the abrogation of LPS-induced acute lung injury by treatment with cANG1-7 *in vivo* (Wösten-van Asperen et al., 2011). These authors further found that cANG1-7 acted very quickly (< 4 hrs) to improve lung function and increase oxygenation. Thus, administration of the modified heptapeptide has a protective role against LPS-induced experimental ARDS. Nevertheless, the ability of cANG1-7 to inhibit acid aspiration-induced and sepsis-induced ARDS also needs to be validated in animal models.

It was also demonstrated that agonists of the Mas receptor or angiotensin type II receptor may hold therapeutic benefits against chronic lung disease (CLD) by counterbalancing ANGII induced pulmonary inflammation. Wagenaar et al. demonstrated cardiopulmonary effects by examination of lung and heart histopathology in neonatal rats challenged with

constant exposure to 100% oxygen for 10 days in the presence of cANG1-7 or an AT2 agonist. Additionally, mRNA levels of crucial genes that are involved in the ANG system and alveolar development were evaluated. Treatment with the agonists reduced the influx of macrophages and neutrophils into the lungs. However, treatment with the agonists did not affect alveolar development in neonatal rats with CLD (Wagenaar et al., 2013).

A non-peptide compound AVE 0991 (AVE) has also shown to mimic the beneficial effects of ANG1-7 in a murine model of ovalbumin (OVA)-induced chronic allergic lung inflammation. Mice were challenged with OVA in the presence or absence of AVE (Rodrigues-Machado et al., 2013). While OVA increased airway and pulmonary vascular wall thickness, OVA + AVE-treated mice displayed reduced airway wall and pulmonary vasculature thickness. Further, cytokine levels and airway contractile response were also reduced in mice treated with AVE compound. Together, these studies suggested the potential of analogues of ANG1-7 in the treatment of chronic pulmonary remodeling associated with asthma.

The protective effects of ACE-2 and ANG1-7 have also been demonstrated in models of pulmonary hypertension (PH). In animal models of PH, ANGII contributes to pulmonary remodeling and binding of ANGII to AT1 receptor is increased in rats with experimental pulmonary hypertension (Kuba et al., 2006). Moreover, ACE expression *in vivo* is also increased in these animals (Shrikrishna et al., 2012). However, preliminary clinical trials did not have major success in demonstrating beneficial effects of ACE inhibitors or ARBs on COPD-related PH (Morrell et al., 2005). Monocrotaline-induced animal models of PH have revealed that experimental overexpression of ACE-2 can inhibit and reverse the induction in right ventricular pressure, suggesting ACE-2 as a potential therapy (Shenoy et al., 2011; Yamazato et al., 2009).

development of a plant-based oral delivery system, consisting of purified ACE-2 and ANG1-7 bioencapsulated in plant cells, has displayed protection against experimental PH (Shenoy et al., 2014). The bioencapsulation defends against gastric enzymatic degradation and improved systemic absorption from the intestine. Sprague-Dawley rats with monocrotaline-induced experimental PH were treated with bioencapsulated ACE-2 and ANG1-7, which significantly halted the disease progression in these animals. This novel approach of delivery system using transplastomic technology may be beneficial for future treatments of other types of lung injuries, but needs to be investigated further. As discussed, ultimately these experimental studies may provide ideas to develop novel therapeutic strategies to control lung diseases and conceivably other diseases that involve the ANG system.

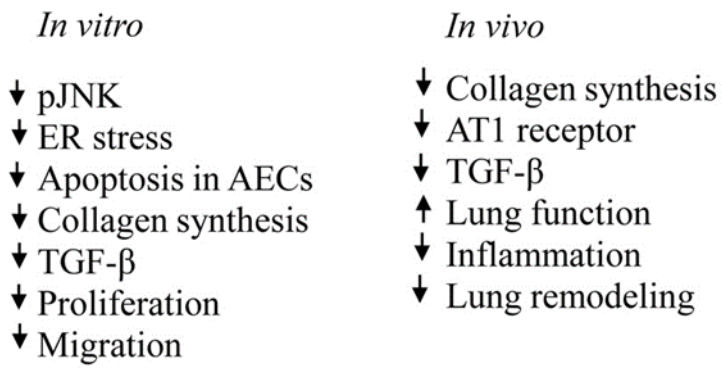


**Figure 1-5: Known actions of ANG1-7 in lung injury.**

In vitro studies of lung fibroblasts have demonstrated the down-regulation of profibrotic genes. Further, ANG II-induced apoptosis of AECs and JNK phosphorylation were reversed by treating the epithelial cells with ANG1-7. In vivo studies have shown the reduction of collagen levels, TGF-β1 and lung inflammation in response to ANG1-7. Moreover, ANG1-7 enhanced lung function in experimental mice after lung injury.

**Signaling Mechanisms Underlying ACE-2/ANG1-7/Mas Action in Lung Cells**

The recent advances discussed above have enhanced our understanding of the tissue specific ANG system and mainly the counter-regulatory role of the ACE-2/ANG1-7/Mas axis which opposes the many deleterious actions of the ACE/ANGII/AT1 axis. However, the exact intracellular signaling mechanisms of the ANG1-7/Mas pathway are currently unclear in lung cells. A number of cell signaling mechanisms are thought to be involved downstream of ANG1-7 binding to Mas, but only a few studies have explored this subject in lung injury. In this section the experimental studies that demonstrate the downstream signaling pathways that are involved in ANG1-7/Mas signaling in lung cells are briefly summarized.

A recent study from the Uhal laboratory demonstrated that ANGII-induced JNK phosphorylation and apoptosis in AECs were potently blocked by ANG1-7 (Uhal et al., 2011). At baseline (without added inducers and in serum-free media), ANG1-7 levels in the medium of cultured AECs are ~10-fold higher than ANGII levels, and thereby function to maintain cell survival (Uhal et al., 2011). The same study demonstrated that ANG1-7 could potently reduce the JNK phosphorylation induced by ANGII, and JNK phosphorylation is required for AEC apoptosis. In accord with these observations, it was hypothesized that ANG1-7 binding to Mas activates a JNK-selective phosphatase which reduces the accumulation of phospho-JNK as a cell survival mechanism. This idea is currently being investigated through the use of gene knockdown strategies. In an earlier study of primary cultures of AECs, it was observed that ANGII binding to AT1 activates PKC and is required for apoptosis (Uhal et al., 2012). Other recent studies showed that blockade of protein kinase A (PKA) by a specific inhibitor led to a rapid increase in pJNK, which suggests a possible role for PKA in AEC apoptosis (unpublished

data). Therefore, it will be of high interest to investigate the possible involvement of the cAMP/PKA pathway in the inhibitory actions of ANG1-7 on lung cells.

Other signaling pathways of ANG1-7/Mas have been reported by various research groups studying lung injury and fibrosis (Figure 1-6). Meng and colleagues showed that the ACE-2/ANG1-7/Mas axis protects against pulmonary fibrosis by inhibiting the MAPK/NF-κB pathway in homogenates of whole lung tissue, thereby reducing markers of fibrosis such as α-collagen-I and synthesis of TGF-β (Meng et al., 2013b). The authors found, in studies of rat lung and in human fetal lung (HFL)-1 cells, that the resistance of fibroblasts to bleomycin- or ANGII-induced apoptosis was prevented by ANG1-7 through inhibition of the MAPK/ NF-κB pathway. Moreover, they found the activation of caspase-dependent mitochondrial apoptotic pathway and BAX protein in response to ANG1-7 in lung fibroblasts. The inhibitory effects of ANG1-7 could be blocked by A779, the Mas receptor blocker, thus showing the involvement of Mas. However, these authors also demonstrated that administration of ANG1-7 alone activated ERK1/2 and moreover, blunted JNK phosphorylation in the HFL-1 cells. The dual effects of ANG1-7 that were shown in this study were explained by the state of activation of the ACE/ANGII/AT1 axis and a possible mechanism of ANG1-7 acting through AT1 receptor. However, no data were provided to support the proposed mechanism of ANG1-7 action through AT1 rather than Mas, and thus this concept should be evaluated carefully, since cell type specificity and the ratio of AT1 vs. Mas receptors are likely to play roles in the activation of this pathway. A later study from the same research group revealed that the ANG1-7/Mas axis protects against fibroblast migration by inhibiting the NADPH oxidase-4 (NOX-4)-derived ROS-mediated RhoA/Rho kinase pathway (Meng et al., 2015).

Recent reports have established the contribution of oxidative stress to the pathogenesis of pulmonary fibrosis (Carnesecchi et al., 2011; Kliment and Oury, 2010). NOX-4 is an important source for the generation of reactive oxygen species (ROS) believed to be involved in initiating lung fibrosis. Consistent with this notion, the induction of α-collagen-I synthesis and fibroblast migration by ANGII was abrogated by an inhibitor of RhoA/Rock pathway (Y-27632) or by siRNA-mediated silencing of NOX-4. A direct inhibitory effect of ANG1-7 was investigated by the ability of ANG1-7 to block ANGII-induced RhoA and Rock-2 mRNA induction. Additionally, the authors showed that lentiviral-mediated expression of ACE-2 suppressed ANGII-induced fibroblast migration and collagen synthesis by blockade of the RhoA/Rho kinase pathway. In agreement with this study, another group showed that ANGII-induced human airway smooth muscle cell (HASMC) contraction was reversed by ANG1-7 through the RhoA/Rho kinase signaling pathway (Li et al., 2012). In this study, HASMCs that were isolated from main bronchus biopsies obtained from lung resection donors were incubated with a RhoA/Rho kinase inhibitor Y-27632, which blocked ANGII induced HASMC contraction. These studies clearly demonstrate a down-regulation of RhoA/Rho kinase signaling pathways by ANG1-7 acting through its receptor Mas. However, further studies are required in other cell types in the lung and to identify the factor(s) that may influence this pathway.

Hashim et al. studied bronchoalveolar lavage fluid (BALF) in allergen-challenged mice and demonstrated that ovalbumin increased total cell numbers of neutrophils, eosinophils and lymphocytes, but this was attenuated by ANG1-7 through suppression of ERK1/ERK2 (El-Hashim et al., 2012). Although the exact mechanism of the suppression is unknown, ANG1-7 attenuated the ovalbumin-induced phosphorylation of ERK1/ERK2 and IκB-α, all of which was prevented by

pre-treatment with the Mas receptor antagonist A779 (El-Hashim et al., 2012). Transcriptional regulation of the ANG1-7/Mas pathway is poorly understood in any organ system. To date, only one study has reported transcriptional regulation (or at least partial regulation) of the ANG1-7/Mas pathway in A549 cells (Verano-Braga et al., 2012). Forkhead box protein O 1 (FOXO-1) is a transcription factor that regulates cell growth and apoptosis; when treated with ANG1-7, FOXO-1 transcriptional factor in A549 cells was phosphorylated and translocated to the nucleus upon stimulation (Verano-Braga et al., 2012). However, further studies are required to understand the regulation of this system in vivo. Several groups have shown the involvement of vascular endothelial growth factor (VEGF), cyclooxygenase (COX-2) and PI3K/AKT pathway in A549s and demonstrated the ability of ANG1-7 to prevent tumor angiogenesis (Menon et al., 2007; Ni et al., 2012; Soto-Pantoja et al., 2009). Nevertheless, further study is needed to determine the role of these pathways in non-neoplastic lung injury.

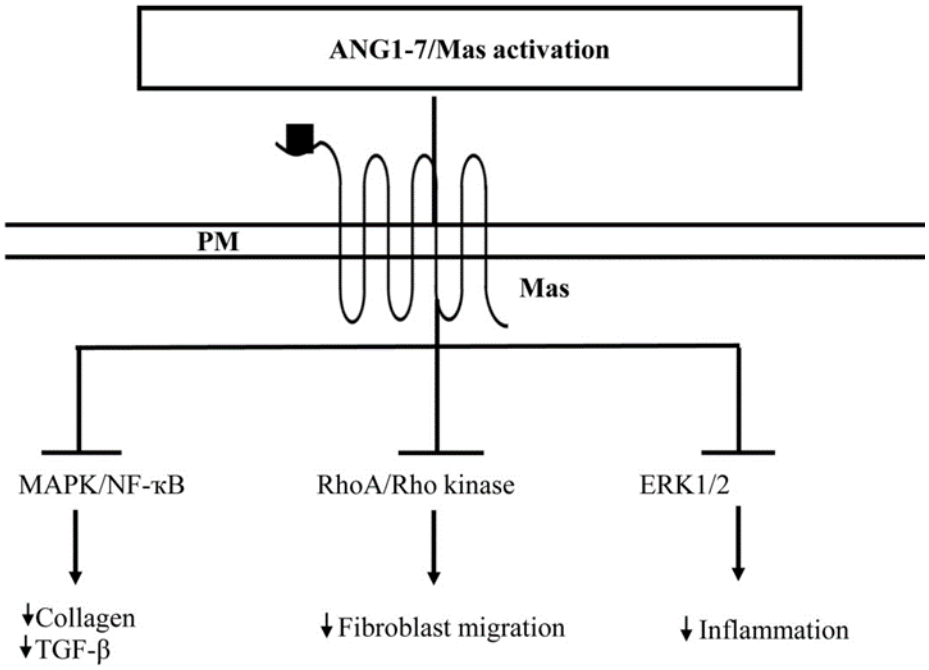


**Figure 1-6: Downstream signaling pathways of the ANG1-7/Mas pathway in non-epithelial lung cell types.**

In lung fibroblasts (Left), ANG1-7/Mas protects against bleomycin-induced lung fibrosis by inhibiting the mitogen-activated protein kinase (MAPK)/NF-κB pathway. Fibroblast migration (Middle) was prevented by inhibition of the RhoA/Rho kinase pathway. Ovalbumin-induced infiltration of lung tissues by eosinophils, lymphocytes and neutrophils (Right) were prevented by ANG1-7 through suppression of ERK1/2. PM – plasma membrane.

**ACE-2/ANG1-7/Mas in Non-Pulmonary Cells**

In the heart, many actions of ACE-2 and ANG1-7/Mas are described in cardiac myocytes. Mas or ACE-2 deficient mice have exhibited a decrease in cardiac contractile which was rescued by administration of ANG1-7 (Bader, 2013). Although, the exact mechanisms by which ANG1-7/Mas abolishes ANGII/ATI are currently unclear, a role of ANG1-7 mediated release of nitric oxide production was reported. Significant induction in cardiac output by ANG1-7 in Wistar rats were prevented by the Mas receptor blocker, A779 demonstrating the importance of Mas in heart (Ferreira and Santos, 2005). Recently, many studies indicate a cardio-protective role of ANG1-7 in the heart through anti-remodeling effect in different models of cardiomyopathy (Santos, 2014). Further, Ferreira et al. demonstrated a significant reduction in cardiac arrhythmias in isolated rat heart in response to ANG1-7. Additionally, in cardiac fibroblasts ANGII induced hypertrophic effects were prevented by ANG1-7/Mas activation (Stewart et al., 2008).

It was shown that ANGII induced kidney damage was reversed by a recombinant form of ACE-2. Further, ANG1-7 infusion or a Mas agonist has shown to ameliorate renal damage in rats. The mechanisms involving these beneficial effects of ANG1-7/Mas, appear to be due to a reduction in oxidative stress (Bader, 2013). Either of ACE-2 gene therapy or delivery of recombinant ACE-2 seem to be protective in diabetic nephropathy. Whether this protective effect is due to an increase in degradation of ANGII or a production of ANG1-7 remains unclear in the kidney (Zimmerman and Burns, 2012).

The brain is one of the organs with highest Mas expression. Depending on the brain area that is under investigation, ANG1-7/Mas have shown to regulate blood pressure. For example,

overexpression of ACE-2 in the medulla decreased blood pressure. ANGII induced reduction of ACE-2 mRNA and protein was prevented by an ATI receptor antagonist in cerebellar or medullary astrocytes in neonatal rat (Xu et al., 2011). *In vitro* experiments have shown ANG1-7 induces vasopressin release and prostaglandin-releasing activity which promotes neuronal activity in the hypothalamus (Xu et al., 2011).

A protective role by ACE-2/ANG1-7/Mas has also been noted in the endothelium. Overexpression of ACE-2, generates ANG1-7 and improves endothelial function in hypertensive rats (Rentzsch et al., 2008). Further, ANG1-7 treatment increased release of nitric oxide and improved endothelial function in Mas transfected Chinese hamster ovary (CHO) cells (Ferreira et al., 2012).

**Novel Therapeutic Targets of ACE-2/ANG1-7/Mas**

In a recent human study, administration of a recombinant form of ACE-2 intravenously showed a decrease in ANGII in the plasma. Results from this study demonstrated the high possibility of cardiovascular protective effects of recombinant ACE-2 (Jiang et al., 2014). In support of this theory, a recombinant form of ACE-2 is currently in clinical trials to treat acute lung injury (Imai et al., 2005). Similarly, an ACE-2 activator, named as XNT was identified and has revealed to decrease blood pressure in hypertensive rats with improved cardiac function (Ferreira et al., 2012). Activation of ACE-2 by XNT increased ANG1-7 levels and co-administration of A779 abolished the ANG1-7 effects.

The therapeutic potential of ANG1-7 is limited due to its short half-life. To circumvent this problem a cyclic form of ANG1-7 has been introduced to produce a bio-stable peptide

analogue. It has demonstrated improved resistance to ACE, increased half-life and most importantly, it exerted Mas dependent vasodilator effects in isolated rat aorta (Jiang et al., 2014; Kluskens et al., 2009). AVE 0991, a non-peptide, is the first small molecule agonist that was discovered and has shown to bind to the Mas receptor with high affinity. This compound has shown to mimic ANG1-7 in the heart, kidney and vessels (Ferreira et al., 2012). AVE 0991 has recently reported to protect against cardiac dysfunction induced by isoproterenol (Iusuf et al., 2008).

Another method to deliver ANG1-7 is through liposomal delivery, encapsulated in lipid vesicles to protect against degradation. Injection of liposome containing ANG1-7 into rats exhibited prolonged hypotension compared to control animals (Ferreira et al., 2012; Iusuf et al., 2008). In addition, development of hydroxypropoyl β-cycledextrin (HPβCD/ANG1-7) has shown significant attenuation of impaired cardiac functions and cardiac remodeling (Ferreira et al., 2012).

## MITOGEN ACTIVATED PROTEIN KINASE (MAPK) SIGNALING

### C-jun-N-terminal kinase (JNK) signaling

Cells respond to various stimuli including temperature changes, pH, growth factors, cytokines, hormones, stress and other chemical stimulations (Cui et al., 2007). The mitogen activated protein kinase (MAPK) family is one of the major signaling systems that consist of protein kinases that regulate signals from outside the cell to activate many intracellular signaling cascades. After the activation of MAPKs, they phosphorylate many proteins that affect the function of the cell. Activation of MAPKs lead to physiological processes including cell

proliferation, differentiation, apoptosis, stress responses and development (Dickinson and Keyse, 2006). MAPKs are activated by dual phosphorylation on threonine (thr) or tyrosine (tyr) residues by upstream MAP2Ks (MAPK kinase), which are activated after phosphorylation by MAP3Ks (MAPK kinase kinase). There are three major classes of MAPKs; extra cellular regulated signaling kinase (ERK) 1 and 2 were the first MAPKs identified. In the following years JNK and p38 MAPKs were discovered. JNK is also recognized as stress activated protein kinases that play a key role in apoptosis. There are three JNK genes in mammals; JNK1, JNK2 and JNK3. JNK1 and JNK2 are ubiquitously expressed where as JNK3 is limited to the central nervous system (Cui et al., 2007). JNK is phosphorylated in the thr or tyr residues of the TXY motif by upstream protein kinases MKK4/MKK7 that are activated by mixed lineage kinases (MLK) including ASK, TAK and MEKK. MKK7 has been shown to activate JNK with high affinity whereas MKK4 can activate both JNK and P38 MAPKs (Dhanasekaran and Reddy, 2008). Deletion of MKK4 in mice, prevented the activation of JNK and reduced cardiac hypertrophy. Studies performed *in vitro* have shown MKK4 phosphorylates JNK on the tyr residue and may be required for optimal activation of JNK (Haeusgen et al., 2011). MKK7 phosphorylates JNK on the thr residue which is vital to trigger JNK activity. cFLIP, an anti-apoptotic protein has shown to bind to MKK7 and inhibit JNK activation.

Phosphorylated JNK can activate downstream targets including c-jun, c-fos, ATF by phosphorylating ser/thr residue or by modulating pro-apoptotic proteins by phosphorylation. Upon activation, JNK can translocate into mitochondria and mediate the release of cyt c to form the apoptosome (Chauhan et al., 2003; Dhanasekaran and Reddy, 2008). Although the exact mechanisms of cyt c release by JNK is unclear, studies have shown a role of pro-apoptotic

protein Bid in the process where it can activate Bax. Also the activation of Bid leads to release of apoptotic proteins from the mitochondria that could induce apoptotic signaling (Dhanasekaran and Reddy, 2008). In line with this, JNK also interacts with other pro-apoptotic proteins such as Bim and Bmf (Lei and Davis, 2003). During UV stimulated apoptosis, the phosphorylation of Bim and Bmf by JNK, release them from hold and activate Bax and Bak in HEK293 cells. Activated JNK also has shown to specifically phosphorylate a serine residue on Bad, one of the pro-apoptotic proteins. Typically pro-survival kinases such as Akt and PKA inhibit Bad by phosphorylating at a ser residue. Activation of JNK, phosphorylates ser at a different position than pro-survival factors do and promote apoptosis in rat neuronal cells (Dhanasekaran and Reddy, 2008).

In AECs, JNK phosphorylation is a required event in apoptosis. It was shown ANGII induced pJNK was blocked by ATI selective antagonist. Inhibition of pJNK by a selective JNK inhibitor prevented the caspase-9 activation by bleomycin (Uhal et al., 2011). Similarly, Chandel et al. demonstrated in mouse lung epithelial cells that bleomycin-induced activation of Bax was abrogated by the expression of a dominant negative JNK. Bleomycin exposed both MLE-12 cells and in primary rat alveolar type II cells, prevented cell death by the dominant negative JNK (Lee et al., 2005). Collectively, these data indicate that JNK dependent activation of cell death in lung epithelial cells.

**JNK Pathway and Diseases**

Unusual activation of JNK pathway has shown to contribute to pathological conditions. Acute respiratory distress syndrome is a severe form of acute lung injury (ALI) and endothelial

cell injury has shown to contribute to ALI. Apoptosis signal-regulating kinase (ASK)-1 is a ubiquitously expressed MAP3K that activates JNK. Knockdown of ASK-1 has shown to reverse caspase activation, JNK phosphorylation and apoptosis in endothelial cells (Li, 2004). Oxidative stress has shown to contribute to lung injury as well, through signaling mechanisms that activate JNK and phosphorylation of its downstream targets including c-jun and activating transcription factor (ATF-1, Li, 2004).

Endothelin (EL-1) is mainly generated in endothelial cell but also is produced in mesenchymal cell types (Abraham, 2008). The receptors for EL-1 are widely expressed in tissues and abnormally high levels are reported in lung diseases. Elevated levels of EL-1 in pulmonary blood vessels and macrophages have been detected in lung injury. Experimental studies have shown TGF-β induces EL-1 in fibroblasts through a JNK dependent mechanism and additionally, JNK activation is reduced when the EL-1 receptors were inhibited (Abraham, 2008).

Some studies have implicated a role of JNK in several cancer types. Recently, experimental data have suggested a different role in function of JNK in normal and tumor cells. Antisense oligonucleotides mediated knockdown of JNK showed to inhibit stress induced apoptosis whereas knockdown of JNK prevented the tumor growth (Davis, 2000). Although the exact role of JNK in tumor development is controversial, JNK was shown to induce hepato-carcinogenesis and may relate on its ability to stimulate cell proliferation. Mutation of JNK phosphorylation sites prevented the tumor size and number (Weston and Davis, 2002). Whether the induction of JNK leads to apoptosis or survival mechanisms seem to depend on the interactions of JNK with signaling pathways (Davis, 2000).

Liver samples from patients with both acute liver injury and fatty liver disease, had high levels of JNK activity. Mice exposed to carbon tetrachloride (CCL4) exhibited elevated levels of JNK activity and mice deficient in JNK prevented liver injury. Further, *in vitro* studies demonstrated JNK inhibitors prevented platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β) signaling in hepatic stellate cells (HSC). Further, the authors demonstrated, angiotensin II-induced HSC activation was prevented by JNK inhibitors by reducing TGF-β and PDGF levels (Kluwe et al., 2010).

**Map Kinase Phosphatases (MKPs)**

MAPKs play a vital role in determining duration and magnitude of physiological signaling. The activity of MAPK signaling is regulated by the activation of MKP that remove the phospho group and limit the actions of MAPK signaling. Thus, MKPs form a signaling complex to negatively regulate MAPK signaling (Owens and Keyse, 2007). MKPs are a group of proteins that belong to the dual specific phosphatase (DUSP) family which consists of ten catalytically active proteins. The MKPs are capable of dephosphorylating both threonine/serine and tyrosine residues of MAPKs. MKPs can recognize and bind to different MAPKs with high specificity but the interaction depends on cell type, cellular localization and response to extracellular stimuli (Lawan et al., 2012).

MKPs contain a N-terminal non catalytic domain and C-terminal catalytic domain. The N-terminal domain consists of two short regions that are homologous to cdc25 cell cycle regulatory phosphatase catalytic site. There are three amino acids (aspartate, arginine and cysteine) in the catalytic site that are absolutely essential for the catalytic activity. Some MKPs

do contain a PEST sequence which is abundant with proline, serine, glutamate and threonine residues. Removal of the PEST sequence, stabilizes the phosphatase (Theodosiou and Ashworth, 2002). The kinase interacting motif (KIM) consists of positively charged amino acids that bind to negatively charged amino acids in MAPKs (Theodosiou and Ashworth, 2002).

Map kinase phosphatase-2 (MKP-2), a 43 kDa protein is one of the earliest identified MKPs (Cadalbert et al., 2005). It is encoded by the DUSP4 gene and is expressed in a variety of tissues. It belongs to the above mentioned DUSP family and is known to negatively regulate MAPKs by dephosphorylating them. MKP-2 has been shown to dephosphorylate JNK selectively *in vitro* and interaction with JNK has shown to increase the catalytic activity. Mouse embryonic fibroblasts derived from a DUSP4 knockout mouse exhibited increased apoptosis and decreased cell proliferation (Lawan et al., 2012). Overexpression of MKP-2 has shown to negatively regulate JNK signaling and prevent JNK dependent apoptosis (Al-Mutairi et al., 2010a). In this study, human endothelial cells were infected with adenoviral MKP-2, which selectively eliminated TNF-α-mediated JNK activation. Further, cellular damage, apoptosis and caspase-3 activation were all reversed by overexpression of MKP-2 in endothelial cells. UV light has shown to induce apoptosis through mitochondrial dependent pathway and overexpression of MKP-2 rescued UV induced apoptosis in embryonic kidney cells 293. Although, more studies *in vivo* are needed to clarify the role of MKP-2, the authors showed that MKP-2 specifically dephosphorylates JNK and a high specificity of MKP-2 for JNK *in vivo* (Cadalbert et al., 2005).

A recent study done by a different group found, $H_2O_2$ induced JNK phosphorylation was abolished by WT-MKP-2 expressing clones in endothelial cells compared to cells expressing a catalytically inactive form of MKP-2. Additionally, activation of ERK1/2 was not prevented by

overexpression of the MKP-2 (Robinson et al., 2001). These data express the importance of phosphatases in regulation of MAPKs which control many signaling pathways.

# CHAPTER 2: ANGIOTENSIN 1-7/MAS INHIBITS APOPTOSIS IN ALVEOLAR EPITHELIAL CELLS THROUGH UPREGULATION OF MAP KINASE PHOSPHATASE-2

**Abstract**

Earlier work from this laboratory showed that autocrine generation of ANGII and pJNK are both required events in AEC apoptosis. Although earlier data showed that ANG1-7 protects against AEC apoptosis, the pathways by which ANG1-7/Mas activation prevent JNK phosphorylation and apoptosis are poorly understood. Therefore, in the current study, it was theorized that ANG1-7 activates a map kinase phosphatase (MKP-2) and thereby reduces JNK phosphorylation to inhibit apoptosis and promote cell survival. This hypothesis was evaluated in both the human and mouse alveolar epithelial cell lines A549 and MLE12, respectively. Cells were transfected with small interfering RNAs, antisense oligonucleotides or inhibitors specific for MKP-2 or Mas, and were then assayed for phospho-JNK, caspase-9, loss of mitochondrial membrane potential (MMP) and nuclear fragmentation. Silencing of MKP-2 significantly prevented the blockade of all apoptotic markers by ANG1-7. Knockdown or blockade of Mas receptor by antisense oligonucleotides or by the receptor antagonist A779, respectively, caused significant decreases in MKP-2, and simultaneously increased the apoptotic markers of caspase-9 activation and nuclear fragmentation. These data show that the ANG1-7/Mas activation prevents JNK phosphorylation and apoptosis by constitutively activating the JNK-selective phosphatase MKP-2, and further demonstrate the critical role of the ANG1-7 receptor Mas in AEC survival.

**Introduction**

It is well established that AEC apoptosis contributes to the pathogenesis of lung injury (Li, 2004). Understanding the underlying signaling mechanisms of AEC apoptosis is critical to determine the pathogenesis interstitial lung diseases (ILD). Blockade of apoptosis by broad spectrum caspase inhibitors or genetic deletion of apoptotic genes prevented lung injury in animal models (Tallant et al., 2005). In recent years, activation of a local ANG in the lung has shown to play a major role in AEC apoptosis and subsequent lung fibrosis (Uhal, 2008). Previous work from this laboratory demonstrated that inducers of apoptosis generate AGT, the 58 kDa protein which, after enzymatic cleavage generates the effector peptide ANGII (Li et al., 2003a; Wang et al., 1999, 2000a). Moreover, it was shown that autocrine generation of ANGII is required in AEC apoptosis, through experiments that blocked apoptosis by either antisense oligonucleotides against AGT mRNA, AT1 receptor antagonists, or by neutralizing antibodies against ANGII itself (Uhal, 2002). Subsequent *in vitro* studies showed that binding of ANGII to its receptor AT1 causes phosphorylation of JNK, a member of the MAPK family, which is required for AEC apoptosis (Uhal et al., 2012).

Recent work showed that ACE-2 is protective against experimental lung fibrosis. Lung tissues from idiopathic pulmonary fibrosis (IPF) patients showed significantly reduced levels of ACE-2 mRNA, protein and enzymatic activity, suggesting that loss of ACE-2 contributes to accumulation of ANGII causing AEC apoptosis and lung injury (Li et al., 2008). Accordingly, in both pulmonary and non-pulmonary systems ANG1-7 has shown to counteract detrimental effects of ANGII through the Mas receptor (Jiang et al., 2014). Radio-ligand binding studies have

provided evidence that ANG1-7 binds to its receptor Mas, which is distinct from the AT1 and AT2 receptor subtypes (Santos et al., 2003).

Experimental studies in this laboratory demonstrated that ANG1-7 inhibits ANGII- or bleomycin (bleo)-induced JNK phosphorylation in AECs (Uhal et al., 2011). Further, ANG1-7 also inhibited caspase activation and apoptosis which were blocked by the Mas receptor antagonist, A779, which has very low affinity for the AT1 or AT2 receptors. Although the exact downstream signaling mechanisms of the ANG1-7/Mas pathway are currently unclear, several groups have shown the activation of a phosphatase in different cell types (Burgun et al., 2000; Gallagher et al., 2008). Map kinase phosphatases are important negative regulators of MAPKs through dephosphorylating the Thr-X-Tyr (T-X-Y) motif of MAPKs (Dickinson and Keyse, 2006). A recent publication by Uhal et al. showed that at baseline (without stimulation) ANG1-7 is more abundant than ANGII in the cell culture media bathing primary AECs and that ANG1-7 dephosphorylates pJNK as a cell survival mechanism (Uhal et al., 2011). Therefore, it was theorized that the ANG1-7/Mas pathway activates a JNK-selective MKP-2 to reduce pJNK levels, thus promoting cell survival. The data herein report the findings that silencing MKP-2 prevents the blockade of JNK phosphorylation and apoptosis (ANGII signaling) by ANG1-7 in AECs. Further, we also report that silencing Mas decreases MKP-2 and promotes apoptosis.

**Materials and Methods**

Reagents and materials: ANGII and ANG1-7 were purchased from Sigma-Aldrich (St. Louis, MO). Mas receptor antagonist A779 (D-Ala7- ANG1-7) was purchased from GenScript (Piscataway, NJ). Antibodies for the detection of MKP-2, Mas receptor as well as MKP-2 specific small interfering RNAs (siRNA) and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for the detection of pJNK, active forms of caspase-9, total JNK, β-actin were all obtained from Cell Signaling Technology (Boston, MA). Antisense oligonucleotides against the Mas receptor and the control antisense were obtained from Genemed Synthesis (San Antonio, TX). 3, 3'-dihexyloxacarbocyanine iodide (DiOC6) was obtained from Life Technologies (Carlsbad, CA). All the other materials were of reagent grade and were purchased from Sigma Aldrich.

Cell culture**:** The human type II epithelial cell-derived cell line (A549) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and was grown in F-12 medium containing 10% serum. The mouse lung epithelial cell line (MLE-12), a generous gift from the laboratory of Dr. J. Whitsett, University of Cincinnati, was grown in complete HITES media. All the cells were grown in 6, 12 or 24 well culture plates and were analyzed at sub-confluent densities. All subsequent incubations with ANG 1–7 and/or A779 (Mas receptor antagonist) were performed in serum-free medium. In all studies cells were exposed to ANG1-7 ($10^{-7}$ M) for 40 min and/or A779 ($10^{-7}$ M) for 30 min before exposure to ANGII ($10^{-7}$ M) for 5 min to 20 h as indicated. Exposure to siRNAs and control siRNAs were done prior to treatment with ANG1-7 and ANGII.

Gene knockdown: Antisense oligonucleotides against human Mas were designed using Antisense design tool from Integrated DNA Technologies (IDT, Coralville, IA) and were synthesized as phosphorothioated 20-mers. A549 cells were transfected with antisense oligonucleotides or control antisense (final conc. 0.1 μM) by using Lipofectamine2000 reagent (Life Technologies, Grand Island, NY) at 2 μl/ml as the vehicle dissolved in F-12 media without any serum or antibiotics. After transfections, the cells were incubated at 37°C with 5% CO2 for six hours followed by addition of normal growth medium with 3 times the normal serum and antibiotic concentration (3x normal growth medium). At 24 h, the transfection reagents were removed and was replaced by complete F-12 media for an additional 24 h. Afterwards, the cells were serum starved overnight and immediately thereafter ANG1-7 ($10^{-7}$ M) was added for 40 min to 12 h as indicated.

The siRNAs against human MKP-2 were commercially synthesized and purchased from Santa Cruz Biotechnologies. The siRNA-to-lipofectine ratios were optimized to yield effective knockdown which was confirmed by Western blotting. A549s were transfected (final conc. 0.1 μM) similarly as described above and were treated with ANG1-7 ($10^{-7}$ M) followed by ANGII ($10^{-7}$ M). A scrambled siRNA of the same sequence was used as a negative control. The MKP-2 siRNA is a pool of three different siRNA duplexes; 1) sense 5'- GAAGGACACUAUCAGUACAtt-3' and antisense 5'-UGUACUGAUAGUGUCCUUCtt-3' 2) sense 5'- GGACUCCGAAUACAUAAUAtt-3' and antisense 5'- UAUUAUGUAUUCGGAGUCCtt-3' 3) sense 5'-CACAGAUCCUAGCAAAUGUtt-3' and antisense 5'- ACAUUUGCUAGGAUCUGUGtt-3'.

Detection of apoptosis: Apoptotic cells were detected by nuclear fragmentation assay using propidium iodide (PI) as described earlier after enzymatic digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5 µg/ml PI (Wang et al., 1999, 2000a). During fixation with 70% ethanol, detached cells were retained by centrifugation of the 24-well culture plates. Cells with discrete nuclear fragments with condensed chromatin were counted as apoptotic using epi-fluorescence microscopy. Apoptotic cells were scored over a minimum of four separate microscopic fields from each of at least three culture vessels per treatment group.

As in earlier publications from this laboratory, the induction of apoptosis is verified by in situ end labeling (ISEL) of fragmented DNA (Uhal et al., 1998). Briefly, the cells were washed with distilled water for 10 min followed by incubation with 0.23% periodic acid for 10 min. After five washes with 0.15 M PBS, cells were incubated with saline-sodium citrate solution at 80°C for 20 min followed by four washes with 0.5 M PBS and 3x with buffer A (50 mM Tris.HCl, 5 mM MgCl2, 10 mM β-mercaptoethanol and 0.005% BSA in water). Next, cells were incubated with an ISEL solution (0.001 mM biotin-dUTP, 0.01 mM of each dATP, dCTP, dGTP, 20 U/ml DNA polymerase I in buffer A) for 2 h at 20°C. Afterwards, cells were washed with 3x in buffer A and with 0.5 M PBS for 5x times. Then the cells were incubated with a Vectastain ELITE solution that contains avidin and biotin-peroxidase solution dissolved in buffer B (1% BSA and 0.5% Tween 20 in 0.5 M PBS). After 30 min, cells were washed with PBS followed by incubation with 0.25 mg/ml diaminobenzidine (DAB) solution in 0.05 M Tris-HCl containing 0.01% $H_20_2$ to detect end labeling. The active forms of caspase-9 were detected by Western blotting using antibodies specific to the cleaved forms.

Estimation of mitochondrial membrane potential: The mitochondrial membrane potential (Ψ, MMP) in A549 cells transfected with MKP-2 siRNA as described above, were assessed with the lipophilic probe DiOC6. After treatment with ANGII ($10^{-7}$ M) for 8 h in the presence or absence of ANG1-7 ($10^{-7}$ M), the cells were incubated with PBS containing 50 nM DiOC6 for 15 min at 37°C followed by an assay in a fluorescence plate reader (BioTek, Winooski, VT) at 360 nm excitation and 420 nm emission. To determine the total DNA, cells were fixed with 70% ethanol for 30 min followed by an incubation with 10 µm Hoechst 33342 dye dissolved in PBS for 10 min. Then the cells were reanalyzed at the same wavelengths for quantitation of total cellular DNA. Data were then normalized.

Western blotting: Cells were lysed either with a modified lysis buffer for phospho-proteins, containing 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1M EGTA, 1.5 mM MgCl2, 100 µM sodium orthovanadate, and the protease inhibitor cocktail (Complete Mini, Roche, Nutley, NJ) or with a Nonidet P-40-based lysis buffer containing protease inhibitors (for MKP-2, Mas and caspase-9). After harvesting, proteins were run on polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Next, the bands were visualized by chemiluminescent substrate West Femto detection systems (Pierce, Rockford, IL).

**Results**

In a previous study, it was found that steady state levels of ANG1-7 levels are much higher in the cell culture media than ANGII levels (Uhal et al., 2011). Thus, it was theorized that ANG1-7 maintains cell survival by dephosphorylating pJNK, by upregulating a map kinase phosphatase (MKP-2). To test this hypothesis, lung epithelial cells were treated with A779 ($10^{-7}$ M) for 30 min after an overnight serum starvation to block the endogenous accumulation of ANG1-7 and cells were harvested to detect pJNK. Figure 2-7A shows that blocking the actions of the endogenous ANG1-7, using the specific Mas blocker A779 added in the final 30 min of a 12 h serum starvation, induces pJNK levels compared to control cells without added A779. To determine if the blockade of endogenous ANG1-7 also reduces MKP-2, AECs were incubated with A779 ($10^{-7}$ M) in similar protocol to the previous experiment. Thereafter, the cells were harvested and subjected for Western blotting. Treating the cells with A779 lead to a reduction in MKP-2 levels in both mouse (Fig. 2-7B) and in human (Fig. 2-7C) alveolar epithelial cells. Further, treatment with A779 ($10^{-7}$ M) reduced MKP-2 levels in primary cultures of human alveolar epithelial cells (Fig. 2-7D) confirming the results from mouse and human cell lines. Further, these data also demonstrate that Mas receptor is functional in primary cultures of human AECs.

Next, to determine whether addition of exogenous ANG1-7 would upregulate MKP-2, the endogenous ANG1-7 was removed after an overnight incubation followed by addition of exogenous ANG1-7 ($10^{-7}$ M) for 40 min. As shown in Figure 2-8, MKP-2 levels were increased in both mouse (2-8A) and human (2-8B) lung epithelial cells compared to the control cells that did not receive exogenous ANG1-7. In Figure 2-9A&B, AECs that received exogenous ANG1-7

showed increased MKP-2 protein compared to the control cells. Further, the induction of MKP-2 by ANG1-7 was blocked by pre-incubating the cells with A779 ($10^{-7}$ M), the Mas receptor antagonist, which demonstrates the involvement of Mas on the induction of MKP-2 by ANG1-7 (in both MLE-12 and A549 cells). Additionally, to show that map kinase phosphatase-1 (MKP-1) and map kinase phosphatase-5 (MKP-5) protein levels did not change with treatment of ANG1-7/A779, cells were treated and harvested similarly as in Fig. 2-9A&B. As shown in Figure 2-9C, the protein levels for both MKP-5 and MKP-1 did not change in response to ANG1-7/A779. As shown previously in Figure 2-9, treatment with A779 reduced the induction in MKP-2 protein compared to the control cells. To confirm this result with antisense oligonucleotides against the Mas receptor, A549 cells were cultured and were treated with antisense in the presence of ANG1-7 ($10^{-7}$ M) for 40 min followed by cell harvesting to detect MKP-2. Silencing the Mas receptor significantly reduced the levels of MKP-2 compared to the cells with ANG1-7 alone (Fig. 2-10) confirming that MKP-2 upregulation occurs through the Mas receptor.

Data from the Figures 2-7, 2-8 &2-9 clearly demonstrated the upregulation of MKP-2 in alveolar epithelial cells. To further investigate the functional roles of MKP-2 in AECs, it was important to determine whether MKP-2 protein can be silenced effectively in A549 cells. After the cells were cultured, they were treated with specific siRNA (final conc. 0.1 μM) against MKP-2. As shown in Fig. 2-11A, treatment with the siRNA silenced the MKP-2 protein compared to the negative control with a scrambled sequence (also compared to transfecting reagents only). To test whether MKP-2 siRNA are specific only for MKP-2 protein and not any of other JNK selective phosphatases, the same samples above were subjected for Western blotting for other known MKPs. Figure 2-11B shows, map kinase phosphatase -3 (MKP-3) and map kinase

phosphatase-7 (MKP-7) protein levels did not change with MKP-2 siRNA. This illustrated that MKP-2 silencing is specific and does not interfere with the expression of the two other known JNK-selective phosphatases MKP-3 and MKP-7, which were unaffected.

Earlier work showed that JNK phosphorylation is a required event in AEC apoptosis (Uhal et al., 2011); to determine whether silencing MKP-2 induces JNK phosphorylation, A549 cells were treated with MKP-2 siRNAs (final conc. 0.1 μM) in the presence or absence of ANG1-7 ($10^{-7}$ M) and /or ANGII ($10^{-7}$ M) for 40 min and 5 min respectively. Figure 2-12 shows that silencing MKP-2 prevented ANG1-7 blockade of JNK phosphorylation. Previously, it was shown that at baseline ANG1-7 levels are protective against AEC apoptosis. Therefore, it was important to access the effects of silencing MKP-2 in the absence of exogenous ANG1-7 and ANGII. To test whether knockdown of MKP-2 increases basal levels of pJNK and caspase-9, cells were treated with MKP-2 siRNA and were harvested after an additional 12 h to detect MKP-2 protein. Figure 2-13A shows the increase in basal pJNK levels when MKP-2 is silenced. Further, silencing MKP-2 also increased the active form of caspase-9 compared to the control cells that did not receive MKP-2 specific siRNA (Fig. 2-13B). Previously, it was demonstrated that the blockade of mitochondrial membrane potential (MMP) loss by ANG1-7 (Uhal et al., 2013b). To determine the effects of silencing MKP-2 could reverse the effects of ANG1-7 on MMP (one of the apoptotic markers), A549 cells were cultured and treated with MKP-2-specific siRNA followed by treatment with the peptides ANG1-7 ($10^{-7}$ M) and /or ANGII ($10^{-7}$ M) for 40 min and 8 h, respectively. A549 cells were then incubated with the lipophilic dye DiOC6 for 30 min and fluorescence was measured. As shown, Fig. 2-14 demonstrates that the loss of MMP induced by

ANGII, was prevented by ANG1-7. Further, the blockade by ANG1-7 was inhibited by siRNA-mediated silencing of MKP-2.

To determine the effects of silencing MKP-2 on inhibition of caspase-9 activation by ANG1-7, A549 cells were cultured and were transfected similarly to the above experiment in Fig. 2-14. Next, cells were treated with ANG1-7 ($10^{-7}$ M) and /or ANGII ($10^{-7}$ M). As shown, in Figure 2-15A, silencing MKP-2 significantly prevented the ability of ANG1-7 to inhibit generation of the cleaved form of caspase-9. Given that, knockdown of MKP-2 increased pJNK, MMP and active form of caspase-9 (Figures 2-12, 14&15A), it was of high interest to determine whether silencing MKP-2 would also increase apoptosis in AECs. To measure nuclear fragmentation (apoptotic body formation) by propidium iodide (PI) assay, A549 cells were cultured and were transfected with MKP-2 specific siRNA followed by incubation with ANG1-7 ($10^{-7}$ M) and /or ANGII ($10^{-7}$ M). Figure 2-15C shows that after 20 h exposure to ANGII, knockdown of MKP-2 significantly prevented the ANG1-7 blockade of nuclear fragmentation induced by ANGII. The results of the nuclear fragmentation assay were also confirmed by in situ end labeling (ISEL) to detect DNA fragmentation using 3, 3'-diaminobenzidine (DAB) method. Again, knockdown of MKP-2 significantly prevented blockade by ANG1-7 (Figure 2-15B). To illustrate that silencing MKP-2 also reverses ANG1-7 signaling in well-differentiated lung epithelial cells, human primary cultures of AECs were cultured and were treated similarly as in Fig 2-15C. As shown in Fig 2-15D, silencing MKP-2 significantly reversed ANG1-7 blockade of ANGII induced nuclear fragmentation, confirming the results with A549 cells.

To investigate the downstream signaling of the Mas receptor in AECs, antisense oligonucleotides (final conc. 0.1 μM) were used to knockdown the Mas receptor, which

significantly silenced the receptor compared to the controls incubated with a scrambled sequence (Figure 2-16A). Next, to determine the importance of Mas receptor in AEC survival, A549 cells were incubated with Mas antisense oligonucleotides, but in the absence of exogenous ANG1-7. The cells were then harvested to detect basal levels of pJNK and caspase. Silencing the Mas receptor significantly induced basal levels of pJNK compared to the control cells (Fig. 2-16B) and similarly, induced the active form of caspase-9 (Fig. 2-16C). These data demonstrated the importance of Mas receptor in AEC survival.

To determine if silencing the Mas receptor induces the active form of caspase-9 in the presence of ANG1-7, A549 cells were treated with Mas antisense oligonucleotides and were treated with ANG1-7 ($10^{-7}$ M) for an additional 8 h. Figure 2-17A shows the increase in active form of caspase-9 when Mas receptor is silenced. Similarly, the cells were cultured and were treated with ANG1-7 ($10^{-7}$ M) for an additional 12 h. Nuclear fragments assessed with PI assay was significantly increased after the Mas receptor was silenced (Figure 2-17B).

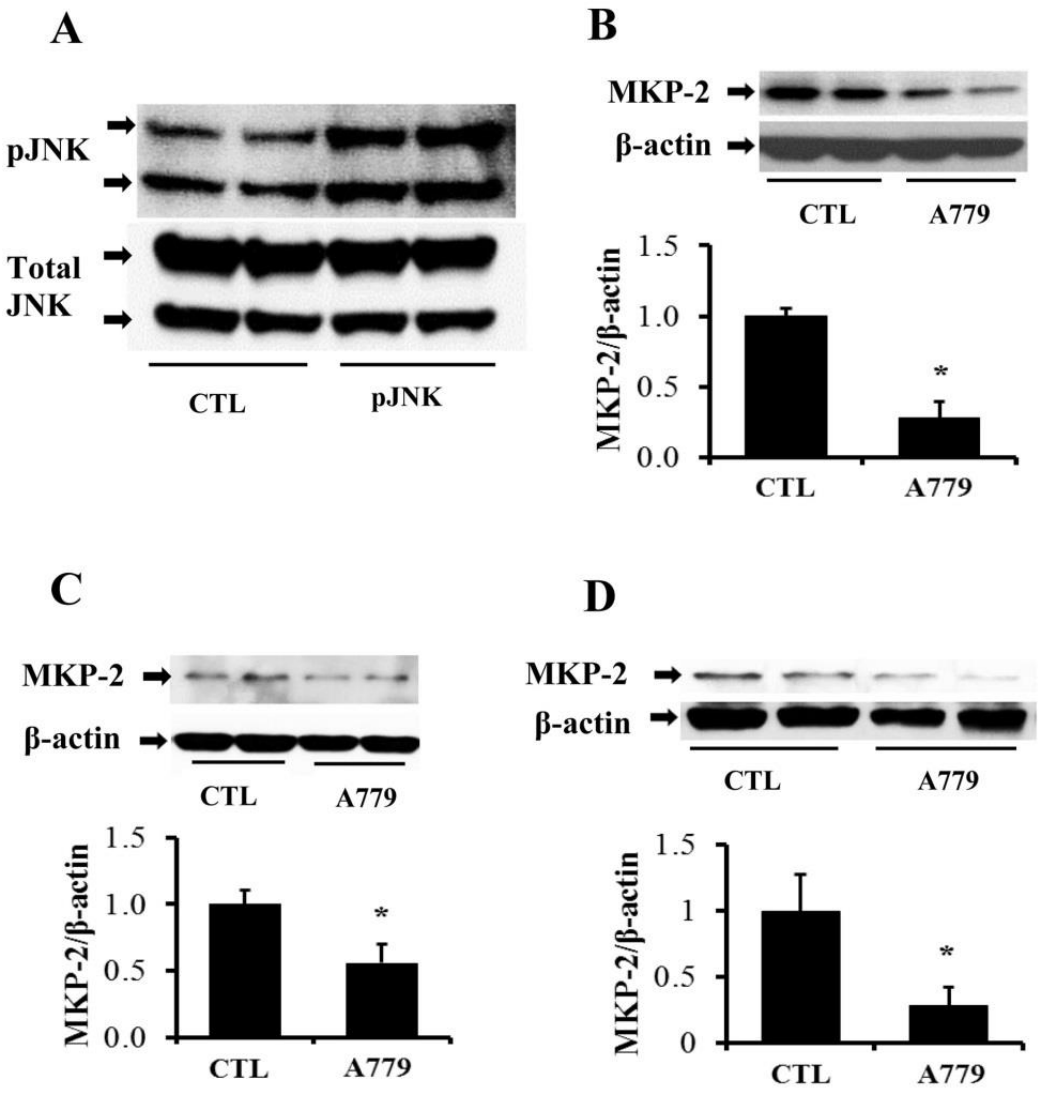


**Figure 2-7: Mas blocker increases pJNK and decreases MKP-2 in lung epithelial cells.**

A) Mouse lung epithelial (MLE) cells were incubated with the Mas receptor antagonist A779 ($10^{-7}$ M) for 30 min after a 12 h serum starvation, without removing the endogenous ANG1-7. Bars are mean + SE of n=4 over two experiments. *$P$ < 0.05 vs. CTL by unpaired t test. B) MLE cells

Figure 2-7 (cont'd)

were cultured and serum starved for 12 h before challenging with A779 ($10^{-7}$ M) for 30 min followed by harvesting for Western blotting. Bars are means + SE of n=4 over two experiments. *$P$ < 0.05 vs. CTL by unpaired t test. C) Similarly, A549 cells were treated as described above in Fig. 2-7B. Bars are means + SE of n=4 over two experiments. * $P$ < 0.05 vs. CTL by unpaired t test. D) Bars are means + SE of n=3 in primary human alveolar epithelial cells. *$P$ = 0.05 vs. CTL by unpaired t test.

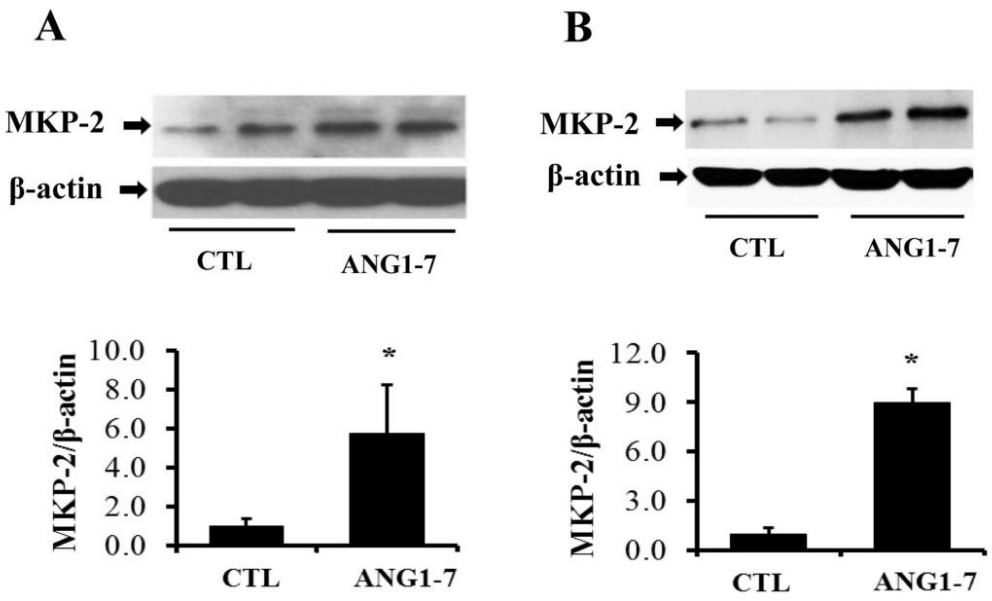


**Figure 2-8: ANG1-7 induces MKP-2 in lung epithelial cells.**

A) MLE-12 cells were serum starved for 12 h, after which the media was removed and were challenged with freshly prepared ANG1-7 ($10^{-7}$ M) for 40 min before harvesting for Western blotting. The control group did not receive ANG1-7. Bars are means + SE of n=4 over two experiments. *$P$ < 0.05 vs. CTL by unpaired t test. B) A549 cells were treated similarly as in above Fig. 2-8A. Bars are means + SE of n=4 over two experiments. *$P$ < 0.05 vs. CTL by unpaired t test.

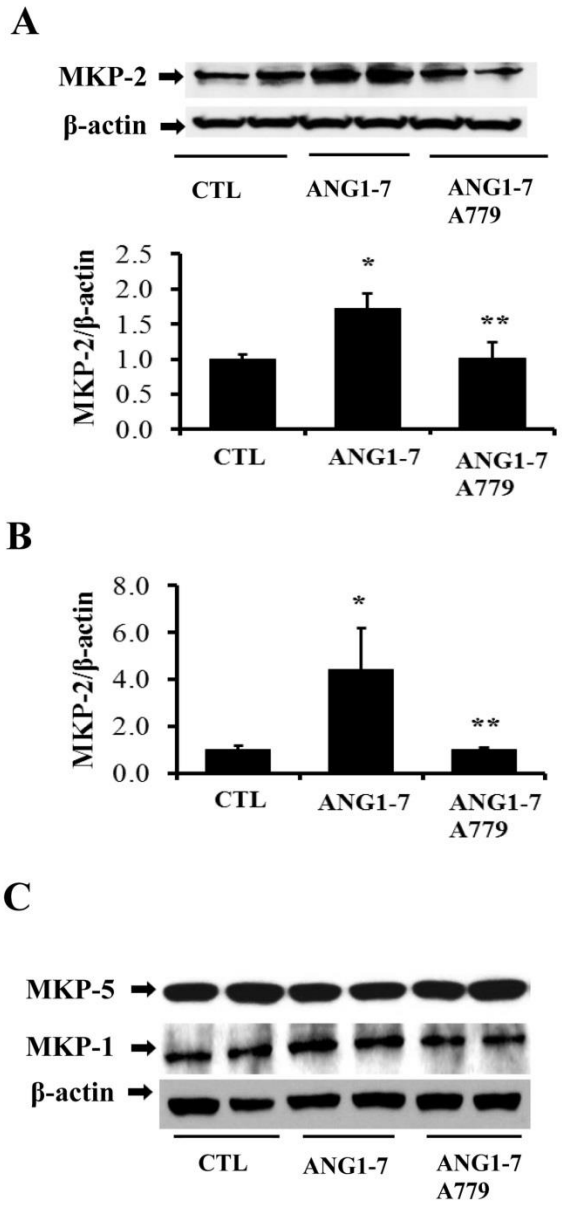


**Figure 2-9: Blockade of MKP-2 induction by a mas receptor blocker.**

A) A549 cells were pre-incubated with ANG1-7 ($10^{-7}$ M) for 40 min in the presence or absence of A779 ($10^{-7}$ M), the mas receptor antagonist followed by cell harvesting for Western blotting. B) MLE-12 cells were treated and harvested similarly as in Fig 2-9A. Bars are means + SE of at least 6

Figure 2-9 (cont'd)

separate cell cultures over two or three experiments.* $P < 0.05$ vs. CTL and ** $P < 0.05$ vs. ANG 1-7 by ANOVA and Student-Newman-Keuls post hoc analysis. C) Same samples as in above were subjected for detection of map kinase phosphatase-5 and 1 as shown.

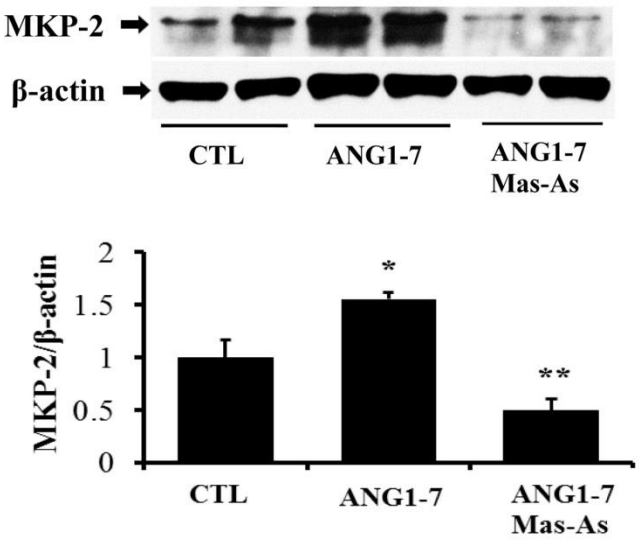


**Figure 2-10: Mas knockdown prevents ANG1-7 induction of MKP-2.**

A) A549 cells were treated with antisense oligonucleotides (final conc. 0.1 μM) in the presence of ANG1-7 ($10^{-7}$ M) for 30 min followed by harvesting. Bars are means + SE of n=4 over two experiments. *$P$ < 0.05 vs. CTL and **$P$ < 0.05 vs. ANG 1-7 by ANOVA and Student-Newman-Keuls post hoc analysis.

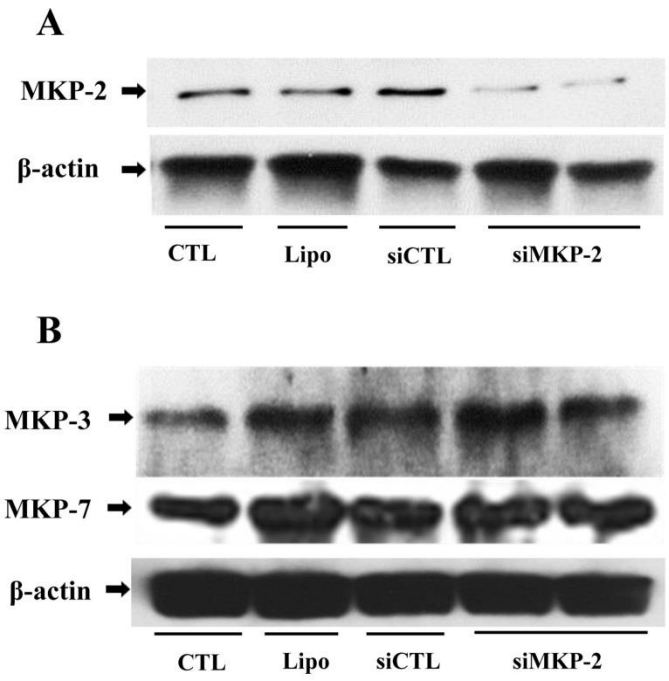


**Figure 2-11: Verification of MKP-2 knockdown by small interfering RNA (siRNA) in A549 cells but not by a scrambled siRNA.**

A) A549 cells were cultured and were transfected with either MKP-2 siRNA or a scrambled sequence using Lipofectamine2000 reagent. After 48 h cells were harvested and subjected to Western blotting. B) The same samples in Fig. 2-11A were used to determine any knockdown effects on other phosphatases (map kinase phosphatase-7 and 3) by Western blotting.

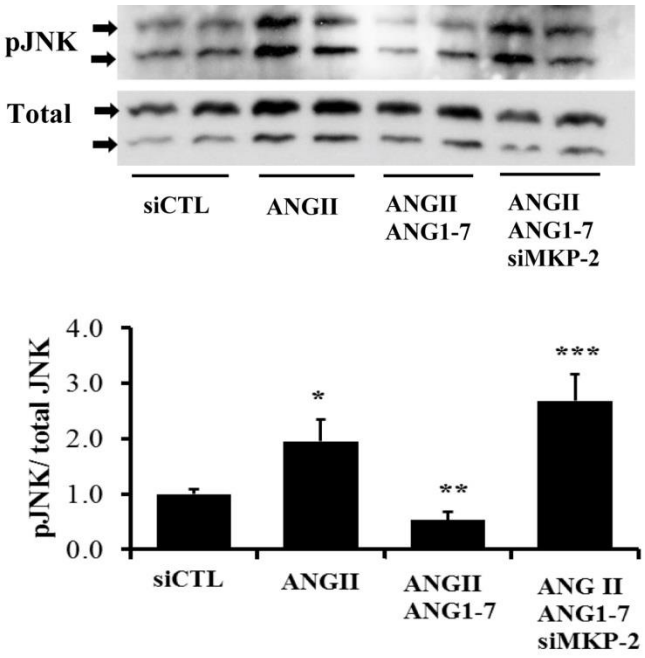


**Figure 2-12: MKP-2 knockdown prevents inhibition of JNK phosphorylation by ANG1-7.**

A549 cells were transfected with MKP-2 siRNA for 48 h, followed by an incubation with ANG1-7 ($10^{-7}$ M) and ANGII ($10^{-7}$ M) for an additional 40 min and 5 min respectively. Next, the cells were harvested for Western blotting. Bars are means + SE of n ≥ 4 over three separate experiments. *$P$ < 0.05 vs. siCTL, **$P$ < 0.05 vs. ANGII and ***$P$ < 0.05 vs. ANGII/ANG1-7 by ANOVA and Student-Newman-Keuls post hoc analysis.

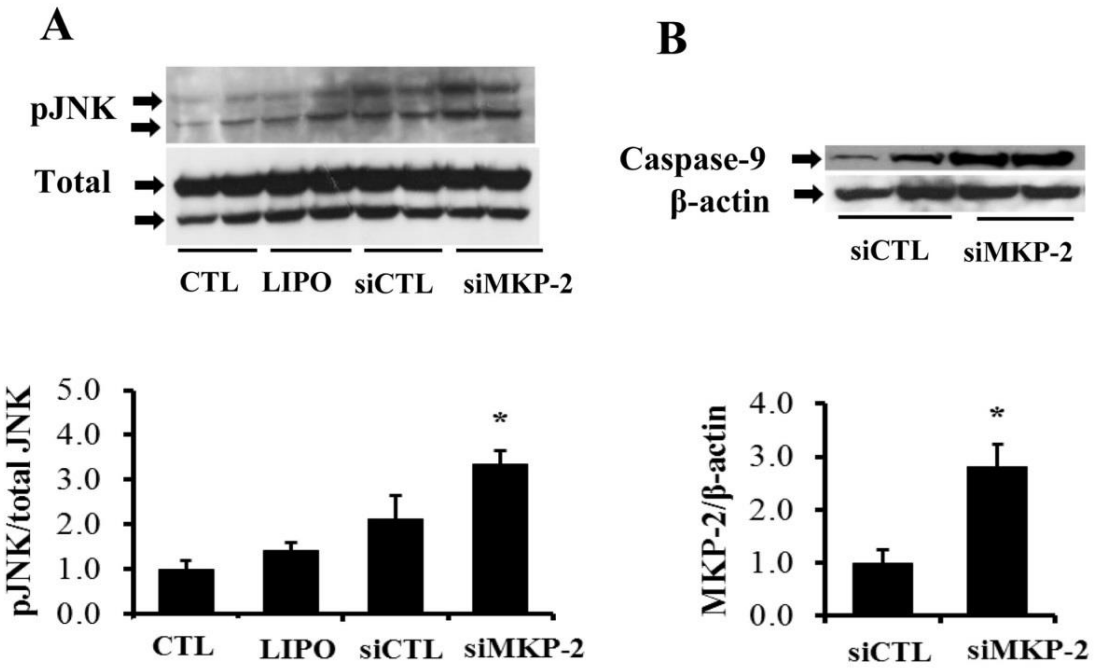


**Figure 2-13: MKP-2 knockdown increases basal pJNK and caspase-9 levels.**

A) A549 cells were treated with MKP-2 siRNA or a scrambled sequence as described in Fig 2-11A. After 48 h, cells were serum starved and were incubated for an additional 12 h. Next, cells were harvested for Western blotting to detect basal levels of pJNK. Bars are means + SE of n ≥ 4 over two experiments. *$P$ < 0.05 vs. siCTL by ANOVA and Student-Newman-Keuls post hoc analysis. B) The same samples above in Fig. 2-13A were subjected for active form of caspase-9 detection. Bars are means + SE of n ≥ 3 over two experiments. *$P$ < 0.05 vs. siCTL by unpaired t test.

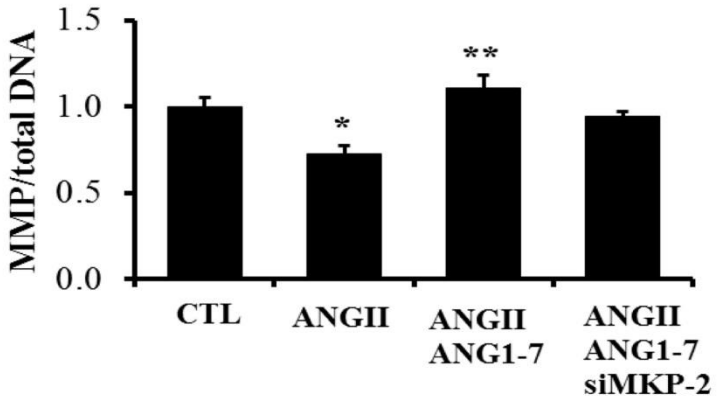


**Figure 2-14: Silencing MKP-2 prevents ANG1-7 rescue of mitochondrial membrane potential (MMP).**

A) A549 cells were cultured in 24 well plates and were treated as in Fig. 2-12 with MKP-2 siRNA and ANG1-7 ($10^{-7}$ M) followed by ANGII ($10^{-7}$ M) for an additional 8 h. Next, cells were incubated with the lipophilic probe 3,3'-dihexyloxacarbocyanine iodide (DiOC6) for 15 min at 37°C for the estimation of mitochondrial membrane potential (MMP). Bars are means + SE of n ≥ 3 in two or more cell cultures. *$P$ < 0.05 vs. CTL and **$P$ < 0.05 vs. ANGII by ANOVA and Student-Newman-Keuls post hoc analysis.

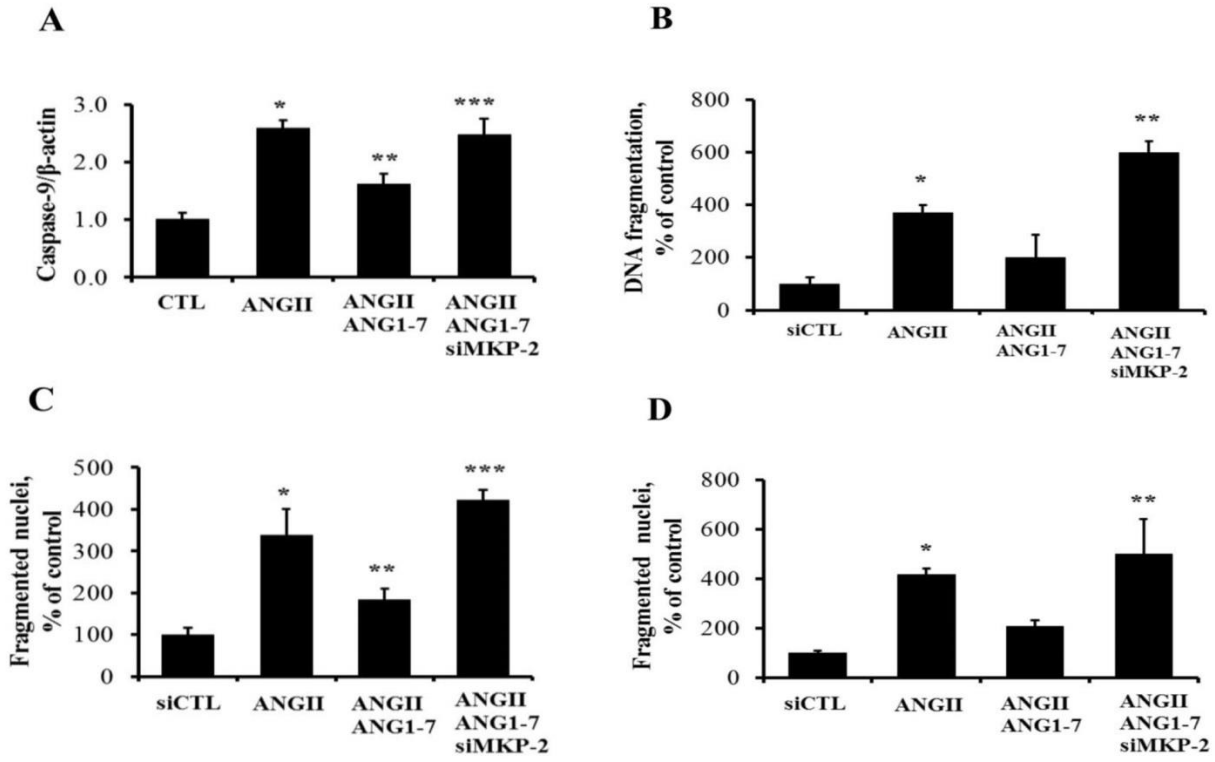


**Figure 2-15: Silencing of MKP-2 prevents inhibition of caspase-9 activation, DNA and nuclear fragmentation by ANG1-7.**

A) After transfection of A549 cells, ANG1-7 ($10^{-7}$ M) was added for 40 min followed by ANGII ($10^{-7}$ M) for an additional 8 h. Next, the cells were harvested for Western blotting and the densitometry bars are means + SE of n=4 over two experiments. *$P$ < 0.05 vs. CTL, **$P$ < 0.05 vs. ANGII and ***$P$ < 0.05 vs. ANGII/ANG1-7 by ANOVA and Student-Newman-Keuls post hoc analysis. B) A549 cells were treated with MKP-2 siRNA followed by incubation with ANG1-7

Figure 2-15 (cont'd)

($10^{-7}$ M) for an additional 40 min. Thereafter, the cells were exposed to ANGII ($10^{-7}$ M) for 20 h followed by fixation of cells and were subjected to in situ end labeling (ISEL) procedure for the detection of fragmented DNA by using 3, 3'-diaminobenzidine (DAB) detection method. Bars are means + SE of n=3; *$P$ < 0.05 vs. CTL and **$P$ < 0.05 vs. ANGII/ANG1-7 by ANOVA and Student-Newman-Keuls post hoc analysis. C) A549 cells were treated as in Fig. 2-15B and were treated with propidium iodide (PI), followed by microscopic quantitation of nuclear fragmentation. Bars are means + SE of n=3; *$P$ < 0.05 vs. CTL, **$P$ < 0.05 vs. ANGII and ***$P$ < 0.05 vs. ANGII/ANG1-7 by ANOVA and Student-Newman-Keuls post hoc analysis. D) Primary human AECs were cultured and were treated with MKP-2 siRNA as in Fig. 2-15C. Bars are means + SE of n=3; *$P$ < 0.05 vs. CTL, *$P$ < 0.05 vs. ANGII/ANG1-7 by ANOVA and Student-Newman-Keuls post hoc analysis.

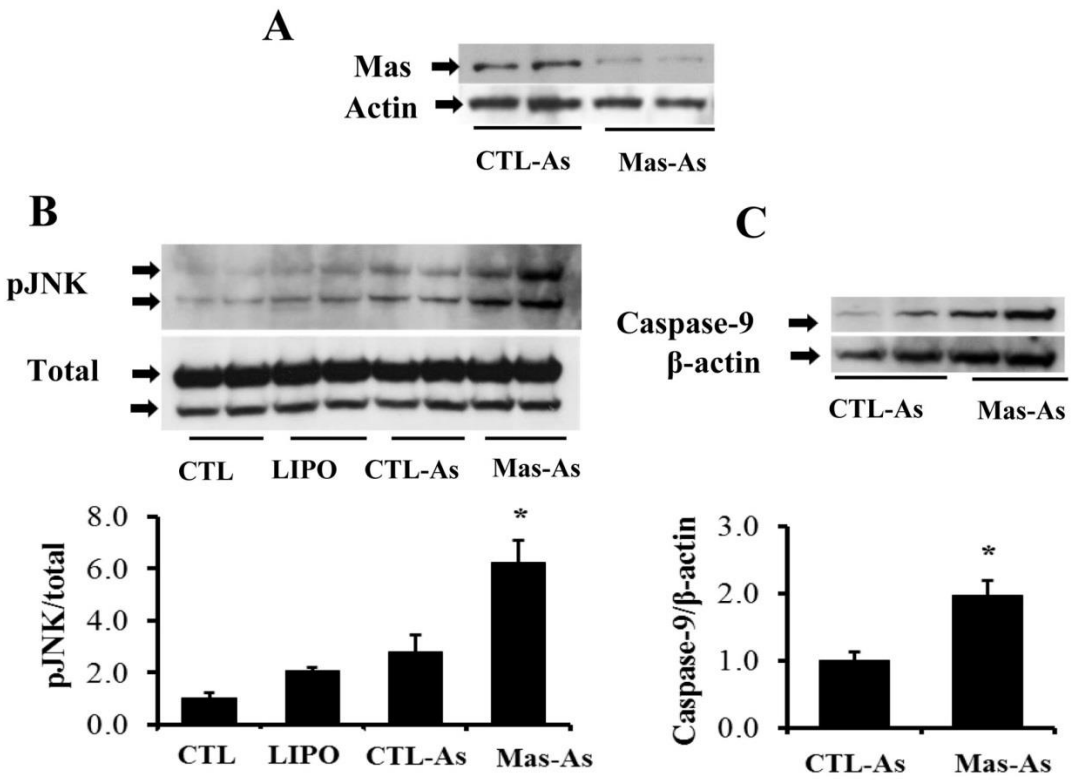


**Figure 2-16: Mas knockdown increases basal pJNK and caspase-9 levels.**

A) A549 cells were treated with antisense oligonucleotides (0.1 µM final concentration) against mas receptor to verify mas knock down. At 48 h, cells were harvested for Western blotting. B) A549 cells were treated with antisense oligonucleotides as described in Fig. 2-16A. At 18 h (post transfection), cells were harvested for Western blotting to detect basal levels of pJNK. Bars are means + SE of n=4 over two experiments. *$P < 0.05$ vs. CTL-As by Student-Newman-Keuls post hoc analysis. C) Same samples above in Fig. 2-16B were subjected for caspase-9 detection. Bars are means + SE of n=4 over two experiments. *$P < 0.05$ vs. CTL-As by unpaired t test.

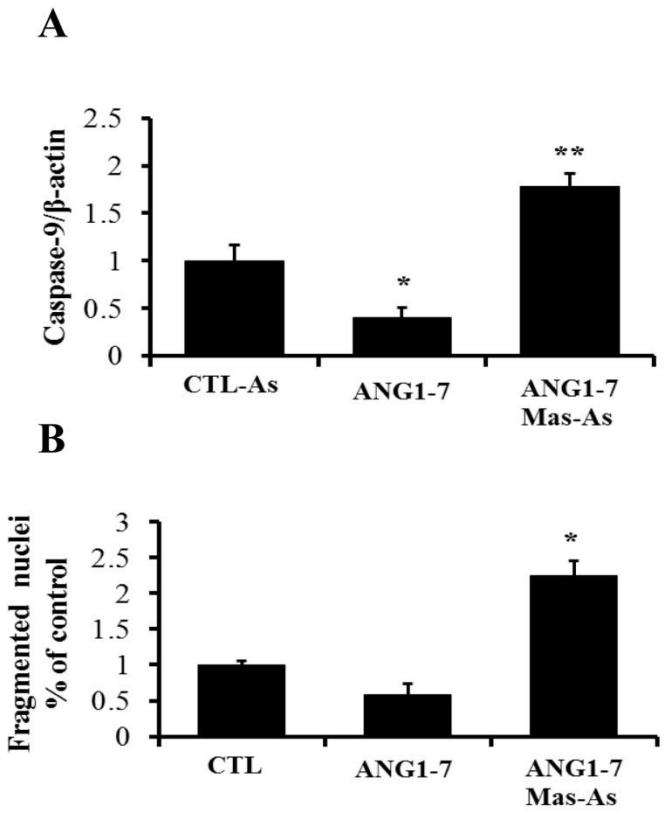


**Figure 2-17: Mas knockdown induces caspase-9 activation and apoptosis in lung epithelial cells.**

A) A549 cells were transfected as in Fig. 2-16A in the presence of ANG1-7 ($10^{-7}$ M) followed by cell harvesting for Western blotting. Bars are means + SE of n=4 over two experiments. *$P$ < 0.05 vs. CTL and **$P$ < 0.05 vs. ANG1-7 by ANOVA and Student-Newman-Keuls post hoc analysis. B) A549 cells were treated with antisense oligonucleotides for 48 h as described above and were subjected to PI assay after 12 h treatment with ANG1-7 ($10^{-7}$ M). Bars are means + SE n ≥ 3 over two experiments. *$P$ < 0.05 vs. CTL and ANG1-7 by ANOVA and Student-Newman-Keuls post hoc analysis.

**Discussion**

The exact downstream signaling mechanisms of the ANG1-7/Mas pathway are currently of high interest to investigators seeking to understand the regulation of AEC apoptosis and its contribution to lung disease. Past studies from this laboratory have shown the involvement of the ANG system in AEC apoptosis and subsequent lung injury (Fine et al., 2000). It is well known that in many organ systems, detrimental effects of the ANGII/AT1 pathway are counteracted by the opposing axis of the ANG1-7/Mas signaling pathway (Iwai and Horiuchi, 2009). Recently, it was demonstrated that JNK phosphorylation is a required event in AEC apoptosis in response to binding of ANGII to the AT1 receptor (Uhal et al., 2011). These authors also showed that bleomycin- or ANGII-induced JNK phosphorylation and apoptosis were blocked by ANG1-7 through its binding to Mas receptor. The enzyme ACE-2 functions as a mono-carboxypeptidase and is one of the enzymes that could degrade the pro-apoptotic ANGII to form the anti-apoptotic ANG1-7 (Hamming et al., 2007). Lentiviral overexpression of ACE-2 has been shown to protect against experimental acute lung injury and cardiac fibrosis in response to bleomycin and ANGII, respectively (Huentelman et al., 2005; Shenoy et al., 2010). Similarly, infusion of ANG1-7 subcutaneously in C57BL/6 mice attenuated lung injury and moreover, treatment with a specific Mas blocker A779 aggravated collagen deposition and lung tissue remodeling (Chen et al., 2013b).

Several studies have shown that imbalance in the levels of extracellular ANGII and ANG1-7 contribute to the pathogenesis of lung injury and defects in other organs (Wösten-van Asperen et al., 2013). A recent study of AECs by Uhal et al. showed that extracellular ANG1-7 levels in serum-free cell culture media are much higher than extracellular ANGII levels under

unstimulated conditions, which was interpreted as a mechanism to maintain cell survival. In the same study it was found that JNK phosphorylation is a required event in AEC apoptosis. Consistent with those findings, in the present study blocking the action of the endogenous ANG1-7 with the Mas antagonist A779 significantly increased JNK phosphorylation (Fig. 2-7A).

Other research groups have shown that in non-pulmonary cells, ANG1-7 inhibits ANGII-induced signaling through activation of a phosphatase. Given that phospho-JNK was increased in response to the Mas blocker (Fig. 2-7A), it was high of interest to determine whether blockade of Mas could reduce MKP-2 protein; this result was observed (Figure 2-7A&B) in AECs. Furthermore, removing the endogenous ANG1-7 and adding freshly prepared exogenous ANG1-7 significantly induced MKP-2 (Fig. 2-8), demonstrating that ANG1-7 regulates AEC survival by upregulating a MAPK-selective phosphatase and dephosphorylating JNK. The further finding that the induction of MKP-2 by ANG1-7 was prevented by the Mas antagonist A779 (Fig. 2-9) showed that ANG1-7 induces MKP-2 through its receptor Mas. These results are consistent with earlier work from this laboratory which demonstrated Mas-mediated blockade of ANGII- or bleomycin-induced signaling by ANG1-7 (Uhal et al., 2011). Together, those data and the results from Fig. 2-9 strongly suggested that MKP-2 mediates its action through the Mas receptor in AECs. The data showing that antisense oligonucleotides against the Mas receptor significantly prevented the ANG1-7-induced MKP-2 protein levels (Fig. 2-10) provide further support for this concept.

Together, these data are consistent with experimental results that several other research groups have obtained by studying non-pulmonary cell types. In proximal tubular cells, ANG1-7 activated a tyrosine phosphatase and thereby prevented high glucose-stimulated

phosphorylation of p38 (Gava et al., 2009). In studies of cardiac myocytes, it was found that ANGII stimulated the phosphorylation of ERK1/ERK2, but this was reduced by cotreatment with ANG1-7 (McCollum et al., 2012b). Moreover, the same authors found that ANG1-7 induces map kinase phosphatase-1 (MKP-1) and further, attenuates cardiac remodeling (McCollum et al., 2012b). Consistent with these findings, transgenic mice with constitutive overexpression of MKP-1, did not activate JNK, p38 or ERK1/ERK2 in the heart and further, catecholamine induced hypertrophy was prevented by overexpression of MKP-1. Those data showed that dual specific phosphatases (DUSPs), primarily MKP-1, are important in counter-regulating MAPKs in cardiac cells (Bueno et al., 2001). Activation of MKP-1 was also demonstrated in vascular smooth muscle cells (VSMCs), and this activation antagonized ANGII/AT1-mediated vascular injury (Takeda-Matsubara et al., 2002). However, the data presented herein strongly demonstrate the upregulation of MKP-2 in alveolar epithelial cells as a cell survival mechanism and treatment with ANG 1-7 and/or A779 did not change MKP-1 or MKP-5 as shown in Fig. 2-9C. These differences could be due to cell type specificity and various signaling mechanisms involved.

In light of the data implicating MKP-2 in AEC survival, it was of high interest to determine the functional effects of MKP-2 silencing in AECs with siRNAs (Fig. 2-11). As illustrated here, knockdown of MKP-2 induced basal levels of pJNK and caspase-9 demonstrating its importance in AEC survival (Fig. 2-13). The siRNA-mediated knockdown of MKP-2 caused a blockade of the ability of ANG1-7 to inhibit ANGII-induced JNK phosphorylation (Fig. 2-12), caspase-9 activation, DNA and nuclear fragmentation (Fig. 2-15). These data are consistent with findings by Gava et al. who showed that blockade of a tyrosine phosphatase by the inhibitor phenylarsine oxide reversed the effects of ANG1-7. Moreover, overexpression of

MKP-2 in human endothelial cells prevented tumor necrosis factor (TNF-α)-induced apoptosis by preventing JNK phosphorylation, and the induction of apoptotic markers by these cells was also reversed by overexpressing MKP-2 (Al-Mutairi et al., 2010b).

A recent publication from this laboratory demonstrated ER-stress induced mitochondrial dysfunction in AECs was blocked by ANG1-7 (Uhal et al., 2013b). In the present study ANG1-7 significantly prevented ANGII-induced reduction of the mitochondrial membrane potential (MMP, Fig. 2-14) and moreover, the prevention by ANG1-7 was blocked in the absence of MKP-2. Cadalbert et al. showed that MKP-2 protects against stress-induced apoptosis in human embryonic kidney cells 293 and moreover, the authors showed specificity of MKP-2 to dephosphorylate JNK *in vivo* (Cadalbert et al., 2005). The data from these groups and our current data suggest that ANG1-7 activates different phosphatases and causes multiple different biological effects in different cell types.

In non-pulmonary cell types, ANG1-7 has shown physiological responses that are opposite to those of ANGII. The hepta-peptide ANG1-7 has been shown to inhibit ANGII-induced MAPK signaling in cardiac myocytes (Tallant et al., 2005), endothelial cells (Sampaio et al., 2007), smooth muscle cells (Freeman et al., 1996) and renal proximal tubular cells (Su et al., 2006). Furthermore, cellular responses to ANG1-7 were blocked by pretreatment with the Mas selective blocker A779. It was shown that Mas knockout mice exhibit impaired cardiac function in vivo and in vitro, which demonstrated the physiologic significance of Mas receptor (Santos et al., 2006). Likewise, the data shown here demonstrate a similar role for the ANG1-7/Mas pathway in AECs; knockdown of the mas receptor, in the absence of ANGII/ANG1-7 showed an induction of basal levels of pJNK and caspase-9 (Fig. 2-16) demonstrating a critical role of mas in

AECs. Moreover, antisense oligonucleotide-mediated Mas knockdown induced the activated form of caspase-9 and nuclear fragmentation as shown in Fig. 2-17. These data confirm the involvement of Mas in AEC survival, and moreover are consistent with previously published data showing that blockade of the Mas receptor with A779 in mouse lung epithelial cells prevented the inhibition of apoptosis by ANG1-7 (Uhal et al., 2011).

To date, only a few studies have investigated the downstream signaling of the ANG1-7/Mas pathway. It was shown that in rat neurons, ANG1-7 induces phosphatase and tensin homolog (PTEN), which dephosphorylates membrane phosphorylated lipids to prevent recruitment of Akt (Modgil et al., 2012). By contrast in isolated adult myocytes, ANG1-7 increased nitric oxide (NO) production associated with induction in endothelial NO synthase and Akt signaling, which were all blocked by treatment with A779 (Dias-Peixoto et al., 2008). These different mechanisms of ANG1-7 action could be due to cell type specificity. Tallant et al. (Tallant and Clark, 2003) demonstrated that ANG1-7 stimulates prostacyclin (PGI2) and stimulated cAMP production in rat VSMCs. However, the molecular mechanisms by which the ANG1-7/Mas pathway stimulates MKP-2 are currently unknown. In vascular smooth muscle cells, blockade of the NO/cGMP pathway prevented the induction of MKP-1 (Jacob et al., 2002). Along similar lines, it was demonstrated that activation of $Na^{+-}$ATPase in response to ANGII was blocked by ANG1-7 through the cAMP/PKA-mediated pathway (Lara et al., 2010). Therefore, it will be of high interest to determine whether cAMP/PKA or cGMP pathways are involved in the induction of MKP-2 in AECs. It is also a possibility that phospho-JNK might be a substrate for other phosphatases, but the results of the siRNA knockdowns shown here suggest that MKP-2 is uniquely responsible for maintaining AEC survival. Although studies have demonstrated the

activation of phosphatases by ANG1-7 in different organs, transcriptional regulation and mRNA stability of the various phosphatases are poorly understood and need to be investigated.

Collectively, the experimental studies herein showed that the ability of ANG1-7 to block ANGII-induced phospho-JNK, caspase-9, MMP, DNA fragmentation and apoptosis is abolished if MKP-2 is silenced. These data support the concept that ANG1-7 upregulates the phosphatase MKP-2 through Mas and thereby maintains low phospho-JNK levels to promote AEC survival. Blockade or knockdown of the Mas receptor by the antagonist A779 or antisense oligonucleotides attenuated the induction of MKP-2 by ANG1-7 and confirmed that Mas acts through MKP-2. These signaling mechanisms suggest the potential for pharmacological manipulation of AEC apoptosis through Mas and MKP-2.

# CHAPTER 3: INVESTIGATION OF THE ROLE OF ANGIOTENSIN 1-7/ACE-2 IN ALVEOLAR EPITHELIAL CELLS DURING ENDOPLASMIC RETICULUM STRESS AND HYPEROXIA

**Abstract**

Previous experimental studies showed that apoptosis of AECs in response to apoptotic inducers is regulated by an ANG system. Autocrine generation of ANGII and its counter-regulatory peptide ANG1–7 have shown to regulate the ANG system in AECs. Endoplasmic reticulum (ER) stress in AECs is a prominent finding in interstitial lung diseases. It was theorized that induction of ER stress causes apoptosis and may also be regulated by the ANG system in AECs. To test this hypothesis, ER stress was induced in MLE-12 cells by the proteasome inhibitor MG132. ER stress induced apoptosis was measured by assays of pJNK, caspase activation, mitochondrial function and nuclear fragmentation. Induction of pJNK by MG132 was significantly inhibited by the non-selective ANG receptor blocker saralasin and was completely blocked by ANG1-7. Hyperoxia is known to contribute to lung injury and ANGII is also involved in experimental hyperoxia induced lung diseases. Both ER stress and hyperoxia decreased the ANGII degrading immunoreactive protein angiotensin converting enzyme-2 (ACE-2). An inhibitor of ADAM17/TACE, significantly reduced ER stress induced reduction of ACE-2 by MG132. Similarly, exposure of human fetal lung fibroblasts (IMR90) to hyperoxic (95% $O_2$/5%$CO_2$) gas reduced ACE-2 immunoreactive protein and enzyme activity. Moreover, soluble ACE-2 protein was increased in the cell culture media, suggesting a role of ectodomain shedding in hyperoxia. These data illustrate that ER stress-induced JNK phosphorylation and apoptosis is regulated by the ANGII and ANG1-7 in AECs. Moreover, ACE-2 is significantly decreased by ER stress/hyperoxic gas through a shedding mechanism mediated by ADAM17/TACE. Further, these data demonstrate ACE-2 and its product ANG1-7 may hold therapeutic strategies for ER stress or hyperoxia induced pathological conditions in the lung.

**Introduction**

Apoptosis of AECs is a critical event that contributes to several lung diseases. Earlier experimental studies demonstrated that apoptotic inducers activate the autocrine synthesis of AGT and its effector peptide ANGII (Li et al., 2003a). Further work illustrated the autocrine production of ANGII and JNK phosphorylation through the ATI receptor are required events in AEC apoptosis (Uhal et al., 2011). More recent studies showed that actions of ACE/ANGII/ATI are opposed by the counter-regulatory axis ACE-2/ANG1–7/Mas which limits the accumulation of ANGII and prevents AEC apoptosis through the ANG1-7/Mas receptor pathway.

In AECs, ER stress can be induced by many deleterious agents that cause apoptosis and subsequent lung diseases (Uhal et al., 2013b; Weichert et al., 2011). Surfactant protein (SP)-C is a type II alveolar epithelial cell specific protein that is synthesized as proSP-C. After subsequent posttranslational processing, the mature SP-C protein is stored in lamellar bodies (Maguire et al., 2012). Mutations in the SP-C protein cause accumulation of misfolded proteins which activate unfolded protein response (UPR). The UPR activates signaling pathways that inhibit protein translation, enhances metabolism, increases protein degradation enzymes and induces the production of chaperone proteins (Lawson et al., 2008). However, prolonged activation of UPR can activate apoptotic signaling pathways and ER stress has shown to induce apoptosis in AECs.

The present study is aimed to investigate the hypothesis that ER stress-induced apoptosis of AECs may also be regulated by the ANG system. To evaluate the hypothesis, AEC apoptosis was induced in MLE-12 or A549 cell line by a synthetic proteasome inhibitor, MG132. The findings herein indicate pro-apoptotic modifications in the ANG system by ER stress and

can be prevented by either blockade of the ANG receptor or by the anti-apoptotic peptide ANG1–7.

In the recent years, experimental studies have also demonstrated that hyperoxia can directly cause lung injury. Damage to the alveolar microenvironment has been reported during hyperoxia and is well characterized in rodents (Pagano and Barazzone-Argiroffo, 2003). It has been reported that exposure of lung tissue to hyperoxia induces reactive oxygen species (ROS) originating in the mitochondria and significantly decreases alveolarization in mice (Ratner et al., 2009). Several reports indicate that hyperoxia-induced lung injury can be prevented by beneficial effects of ACE-2 in neonatal rats (Wagenaar et al., 2013). Further, *in vivo* data suggest that recombinant expression of ACE-2 can prevent lung injury in mice (Imai et al., 2005). Therefore, ACE-2 may play a major role, since the components of the local ANG system is expressed and is functional in human lung myofibroblasts, the primary source of collagen deposition in the lung (Abdul-Hafez et al., 2009; Oarhe et al., 2015).

ANGII, the effector peptide in the ANG system, induces collagen synthesis through the ATI receptor in the lung fibroblasts. Both angiotensin receptor blockers and ACE inhibitors have demonstrated to protect against lung injury (Marshall et al., 2004; Wang et al., 2000b). *In vivo* experimental studies performed in fetal lung fibroblasts exposed to oxygen showed the upregulation of the ANG system (Lang et al., 2010). Prior work from this laboratory, found that ACE-2 is downregulated in response to bleomycin and additionally, a purified recombinant human ACE-2 diminished, bleomycin-induced lung collagen accumulation (Li et al., 2008). Further, it was revealed hyperoxia significantly increased total collagen content in neonatal Sprague-Dawley rats. Moreover, ANG system components including ATI receptor and ACE were

induced in hyperoxia (Jiang et al., 2012). Additionally, hyperoxia induced collagen deposition was blocked by ATI antagonist losartan demonstrating the upregulation of the ANG system in hyperoxia (Chou et al., 2012).

The role of TNF-α converting enzyme (TACE), also known as ADAM17, was evaluated by several groups and demonstrated its ability to cleave the ectodomain of ACE-2 (Haga et al., 2008, 2010). Further, the "shedding" of ACE-2 ectodomain was blocked by the TACE antagonist, TAPI-2. Given the known involvement of the ANG system components in lung fibroblast function it was hypothesized that ACE-2 might be downregulated by hyperoxia in lung fibroblasts and further, downregulation of ACE-2 could be prevented by inhibiting TACE.

**Materials and Methods**

Reagents and materials: The proteasome inhibitor MG132 (carboxybenzoxy-Leu-Leu-leucinal) was obtained from GenScript, Piscataway, NJ. ANG1–7 and the non-selective ANG receptor saralasin was obtained from Sigma Chemical, St. Louis, MO. Antibodies for the detection of phospho-JNK, total JNK, β-actin and secondary antibodies were purchased from Cell Signaling, Danvers, MA. Antibodies for the detection of ACE-2 were obtained from Abcam Biotechnology, Cambridge, MA. Protease inhibitor cocktail and broad spectrum phosphatase inhibitors were obtained from Roche, Nutley, NJ. TAPI-2, an inhibitor of ADAM17 was purchased from Calbiochem, San Diego CA. For sample dialysis, Spectra/por biotech cellulose ester dialysis membranes with a MWCO 10kDa were obtained from Spectrum Laboratories, Rancho Dominguez, CA. All other materials were of reagent grade and were obtained from Sigma Aldrich, St. Louis, MO.

Cell culture: The mouse lung epithelial cell line MLE-12, a gift from Dr. Jeffrey Whitsett, University of Cincinnati, OH, was cultured in complete HITES medium as described previously. All cells were grown in 6-well chambers and were analyzed at sub-confluent densities of 60–80% except where indicated. All subsequent incubations with ANG1-7 ($10^{-7}$ M) or saralasin (50 μg/ml) or other test agents were performed in serum-free medium. In all studies, cells were exposed to antagonists 30 min before exposure to MG132 (10 μM) for 5 min. The fetal lung fibroblast cell line (IMR90) was purchased from ATCC (Manassas, VA) and was cultured in complete media. At confluence fibroblasts were exposed to hyperoxic (95% oxygen with 5% $CO_2$) or normoxic (21% oxygen with 5% $CO_2$) gas for 72 h, in 5% fetal bovine serum. Some cells were also treated with TAPI-2 (10 μmol/L), before exposure to hyperoxia. After 72 h, media on

cells were aspirated and rinsed once with serum-free media. Cells were then allowed to recover in serum-free media for 24 h in room air. At the end of the recovery period, cells were harvested and assayed for ACE-2 protein by Western blotting.

Cell Culture Media Handling*:* Media on cell culture (a total volume of 1 ml) were added to a 15 ml conical tube containing 300 µl of complete protease inhibitor EDTA-free cocktail. After centrifugation to remove cell debris, samples were subjected for dialysis. Next, membranes containing the samples were kept with gentle stirring in a 2L beaker with deionized water at 4°C for two days till samples were clear. Samples were then transferred to a new 15 ml canonical tube and frozen at −80°C and were lyophilized using the Labconco Freeze Dryer/Freezone 4.5 system according the manufacturer's protocol. Samples were diluted in 100 µl of sterile deionized water and ACE-2 protein levels were analyzed by Western blotting.

Western blotting: Following treatment, cells were lysed with 200 µl of modified lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 µM EGTA, 1.5 mM $MgCl_2$, 100 µM protease inhibitor cocktail tablet and phosphatase inhibitor for detection of phospho-proteins. After harvesting, proteins were run on 10% polyacrylamide gels in tris/glycine/SDS buffer and were transferred to PVDF membranes. After transferring the proteins, the membrane was blocked in 5% nonfat dry milk in 0.1% tween 20 in tris-buffered saline (TBS). Western blot analysis of pJNK was performed with stress activated protein kinase (SAPK)/JNK antibody (1:000 dilution) with overnight incubation at 4 °C followed by HRP-conjugated secondary anti-mouse antibody (1:2000) incubation the next day. For the detection of total JNK, the membranes were stripped and re-probed with total JNK antibody.

Immunoreactive bands were visualized by West Femto substrate systems (ThermoScientific, Rockford, IL).

**Results**

Previous work from this laboratory showed, blockade of bleomycin induced JNK phosphorylation and apoptosis by ANG1-7 (Uhal et al., 2011). Additionally, it was found that JNK phosphorylation is mediated by the ATI receptor through experiments that prevent JNK phosphorylation by ATI receptor antagonists. Therefore, the effect of the anti-apoptotic peptide ANG1-7 or the non-selective receptor blocker saralasin on JNK phosphorylation was determined in AECs treated with the proteasome inhibitor MG132. Mouse lung epithelial cells were cultured and were treated with saralasin (50 μg/ml) or ANG1-7 ($10^{-7}$ M) for 30 min followed by treatment with MG132 for an additional 5 min. Next, the cells were harvested. Figure 3-18 shows partial blockade of MG132-induced JNK phosphorylation by saralasin and complete blockade by ANG1-7.

To investigate, whether exposure of the fetal fibroblasts to hyperoxic gas with recovery, increase the cleavage of membrane bound ACE-2 by ADAM17/TACE, cell culture media was collected after exposure of IMR90 cells to hyperoxia (95% $O_2$) in the presence or absence of TAPI-2 (10 μmol/L) and were analyzed for soluble ACE-2 protein. Figure 3-19 shows exposure of IMR90 cells to hyperoxia significantly induced the soluble form of ACE-2 in the culture media compared to the control cells demonstrating the cleavage by ADAM17/TACE. Further, addition of TAPI-2 prevented the induction of soluble ACE-2 by ADAM17.

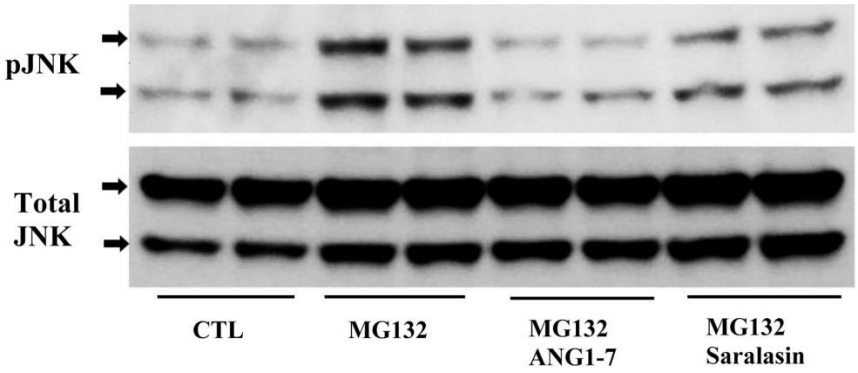


**Figure 3-18: Blockade of ER stress induced JNK phosphorylation by angiotensin 1–7 or by saralasin.**

MLE-12 cells were grown in 6 well culture plates and were serum starved overnight at sub-confluent densities. Thereafter, saralasin (50 µg/ml) or angiotensin 1–7 ($10^{-7}$ M) were applied to cells for 30 min immediately prior to challenge with MG132 (10 µM) for an additional 5 min. Next, cells were harvested for Western blot analysis of phosphorylated JNK. Results are indicative of those obtained in at least 3 separate experiments.

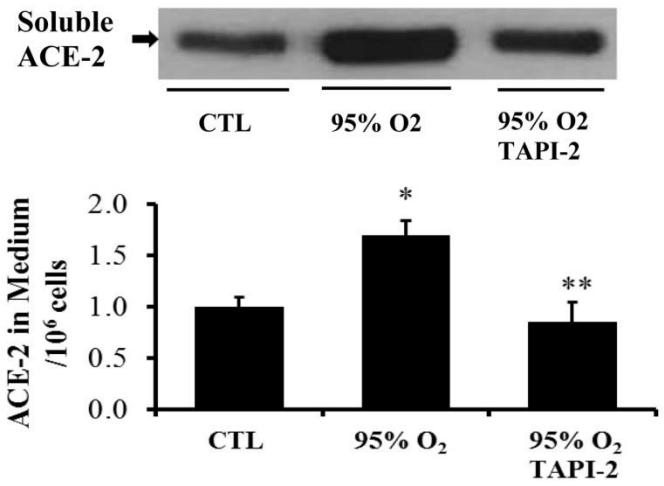


**Figure 3-19: ADAM17 blocker prevented the induction of soluble ACE-2.**

IMR90 cells were cultured and exposed to hyperoxic or normoxic gas as described in method section, in the presence or absence of the ADAM17 inhibitor TAPI-2 (10 µmol/L). Cell-free culture media were then collected separately from the cell monolayer. Lyophilized samples were subjected to western blotting for ACE-2. Bars are means + SE *$P$ < 0.05 vs. CTL and **$P$ < 0.05 vs. 95% O2 by ANOVA and Student-Newman-Keuls multiple comparisons test.

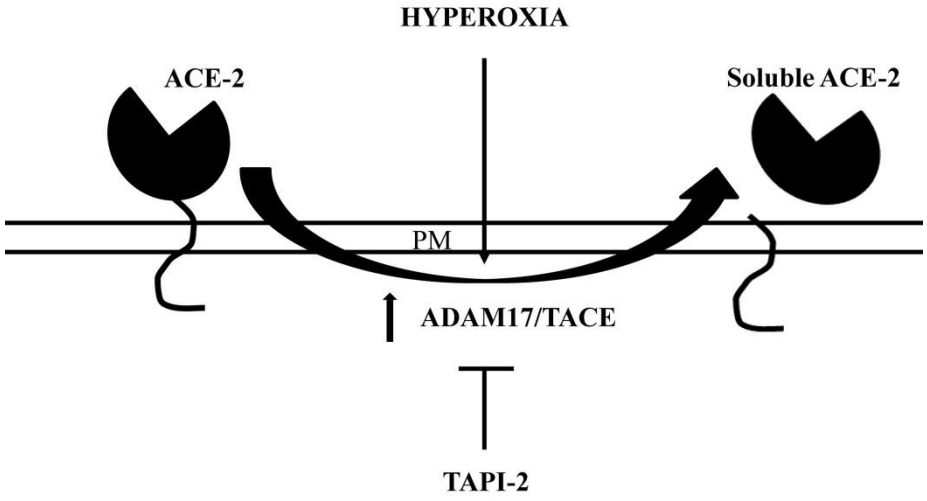


**Figure 3-20: Ectodomain shedding of angiotensin-converting enzyme-2 (ACE-2) in hyperoxia-induced lung injury.**

Hyperoxia induces ADAM17/TACE which in turn mediates the release of the ectodomain of ACE-2 from the cell. Loss of ACE-2 promotes accumulation of ANGII and reduces the production of ANG1-7, contributing to lung injury.

**Discussion**

The notion that induction of apoptosis in AECs is sufficient to induce a pathological microenvironment in the lung is supported by experimental studies that began almost 40 years ago (Uhal et al., 2013). Recent experiments that demonstrated blockade of apoptosis with broad spectrum caspase inhibitors or by deletion of genes that are essential in apoptosis prevented lung injury (Budinger et al., 2006; Kuwano et al., 2001; Wang et al., 2000b). Recently ER stress has shown to induce apoptosis in AECs and contribute to interstitial lung diseases. Accumulation of unfolded proteins in the ER activates UPR which attempts to enhance protein folding. However, overwhelming ER stress may trigger AEC apoptosis (Korfei et al., 2008).

Previous experimental studies from this laboratory showed activation of the ANG system in response to bleomycin, fas ligand and TNF-α which induces the production of AGT and ANGII in the AEC itself (Li et al., 2003a; Wang et al., 1999, 2000a). Further, ANGII or bleomycin induced JNK phosphorylation and apoptosis was blocked by selective ATI antagonists showing AEC apoptosis is mediated through ATI receptor (Uhal et al., 2011). Moreover, addition of AGT to cultured AECs in serum free media increased ANGII receptor-dependent apoptosis demonstrating the constitutive expression of required enzymes in AECs (Li et al., 2004). Recent studies have revealed the regulation of AEC apoptosis by the counter-regulatory axis of the ANG system, ACE-2/ANG1–7/Mas. Constitutive expression of ACE-2 in AECs, limits the accumulation of ANGII by degrading the octa-peptide to ANG1-7 which inhibits AEC apoptosis by decreasing JNK phosphorylation through the receptor Mas (Uhal et al., 2011).

SP-C is solely produced and secreted by the type II AECs. Accumulation of mutant SP-C or protein complexes in the ER has shown to cause injury to AECs (Korfei et al., 2008; Whitsett,

2002). Overexpression of Bcl-2, an anti-apoptotic protein demonstrated blockade of ER stress induced loss of mitochondrial membrane potential (Schröder and Kaufman, 2005). Additionally, prolonged activation of the UPR showed induction of pJNK and its downstream targets (Maguire et al., 2011). Activation of JNK has shown to phosphorylate BH-3 family related proteins and also anti-apoptotic proteins including Bcl-2 in ER stress conditions (Szegezdi et al., 2006). Deletion of apoptosis-signal-regulating kinase (ASK1), a MAP3K that activates JNK, exhibited resistance to ER stress (Nishitoh et al., 2002). Together, these data presented by other research groups may suggest that phosphorylation of JNK by ER stress targets Bcl-2 family proteins and causes dimerization of Bax and Bak leading to apoptosis (Hetz et al., 2006; Tanjore et al., 2013).

The data presented herein, show the regulation of ER stress induced AEC apoptosis by the ANG system. Figure 3-18 shows, MG132 induced JNK phosphorylation was blocked by ANG1-7 and was only partially blocked by saralasin. This result shows a more potent role of ANG1-7 in ER stress induced apoptosis in AECs compared to ANG receptor blockers. Consistent with this finding, a clinical trial demonstrated positive results of ANG receptor blockers but was limited to a smaller number of patients (Couluris et al., 2012; Woo et al., 2003). A purified recombinant ACE-2 or ANG1-7 has already demonstrated to protect against lung injury in animal models (Li et al., 2008; Shenoy et al., 2014).

Recently, it was found that hyperoxia also upregulates the ANG system including ATI receptor expression. Oxidative injury is a well- documented mechanism that is associated with abnormalities in alveolar development (Ratner et al., 2009). Experimental studies have shown proliferation of fibroblasts and differentiation to myofibroblasts, a major source of the

extracellular matrix production in hyperoxia-induced lung injury (Rehan and Torday, 2003). It was shown that siRNA mediated knockdown of the ATI receptor attenuated hyperoxia induced type I collagen expression, suggesting a role of ANGII/ATI in lung fibroblasts (Lang et al., 2010). Earlier work from this laboratory and other studies conducted by different groups, have demonstrated the protective role of ACE-2 in lung injury and a recombinant form of human ACE-2 (rhACE-2) is in clinical trials to treat acute lung injury. However, the role of ACE-2 has not been investigated in the hyperoxic-induced injury in lung fibroblasts.

ACE-2 has been found to play a vital role in virus-induced lung diseases. ACE-2 is the cellular receptor for severe acute respiratory syndrome (SARS-Coronavirus) which is essential for coronaviral entry (Dimitrov, 2003). SARS- Coronavirus spike protein has shown to induce shedding of ACE-2 in lung tissue (Haga et al., 2010). Additionally, ACE-2 has also shown to downregulate in influenza A (H7N9) virus-induced acute lung injury (ALI) and deficiency of ACE-2 aggravated ALI in mice (Yang et al., 2014). Similarly, a downregulation of ACE-2 expression in the lung in response to H5N1 virus resulted in an induction in serum ANGII levels (Zou et al., 2014). Genetic deletion of ACE-2 in H5N1 challenged mice caused severe lung injury and recombinant ACE-2 improved H5N1 induced lung injury confirming a protective role of ACE-2.

ACE-2 is a type I transmembrane protein with a N-terminal ectodomain containing the active site. Ectodomain shedding has been observed in many transmembrane proteins and seems to be mediated by ADAM (a disintegrin and metalloproteinase) protein family. ADAM17 or TNF-α converting enzyme (TACE) was identified as a TNF-α sheddase but has shown to cleave other membrane proteins (Garton et al., 2003; Wang et al., 2002). Inhibition of ADAM17 by siRNA mediated knockdown decreased ACE-2 shedding and overexpression caused increased

shedding, suggesting the role of ADAM17/TACE in ACE-2 shedding (Lambert et al., 2005). In cardiovascular diseases circulating levels of ACE-2 has been reported although the physiological role of shedding remains unclear (Shaltout et al., 2009). In line with this, another group demonstrated ADAM17/TACE mediated ACE-2 ectodomain shedding in the brain which decreased membrane bound ACE-2, thus stimulating the development of neurogenic hypertension (Xia et al., 2013). AECs are the primary site of ACE-2 expression in mice and has been studied extensively demonstrating its protective role against lung injury (Uhal et al., 2011; Wiener et al., 2007). Exposure of Primary cultures of rat AECs to hyperoxic gas caused apoptosis of alveolar epithelial cells (Wang et al., 2014). However, exposure of lung fibroblasts to hyperoxia did not upregulate markers of apoptosis and this might be due to activation of different signaling mechanisms in lung cell types.

In the present study it is shown that TAPI-2, significantly prevented the induction of soluble ACE-2 in response to hyperoxia in the culture media of IMR-90 cells (Fig. 3-19; adapted from Oarhe et al., 2015). It is prominent that the reduction in ACE-2 protein in IMR90 cells occurred only if the hyperoxic exposure was followed by normoxic period of recovery. This is a notable concept that was also seen in ischemia–reperfusion injury, in which many vital signaling cascades are activated during the return to normoxia but not during the ischemic period (Kulkarni et al., 2007).

In summary, the exact signaling mechanisms by which ER stress induce the ANG system to initiate AEC apoptosis are currently unclear but possibly involve both axis of the ANG system (Uhal et al., 2013b). The ability of ANG1–7 to completely abolish JNK phosphorylation suggests that the ANG1–7/Mas pathway is a more effective regulator of ER stress-induced apoptosis. It

was also shown that exposure of human lung fibroblasts to hyperoxic gas seems to involve the upregulation of ADAM17/TACE protein which facilitates the ectodomain shedding of ACE-2 protein (Fig. 3-20). The exact signaling mechanism(s) by which hyperoxia upregulates TACE still remain unclear and need to be elucidated. Together, the data reported here show that both ER stress and hyperoxia upregulate the ANG system and that ACE-2 or its product ANG1-7 may hold the therapeutic potential to treat lung diseases that involve ER stress and hyperoxia.

# CHAPTER 4: SUMMARY AND CONCLUSIONS

## SUMMARY

### Upregulation of Map Kinase Phosphatase-2 in Alveolar Epithelial Cells

Previous work found that apoptosis in AECs requires the local production of angiotensinogen and its effector peptide ANGII (Xiaopeng Li, 2003). Recent studies showed that ACE-2 is protective but severely downregulated in human and experimental lung tissue in response to bleomycin (Li et al., 2008). Earlier experimental studies from this laboratory demonstrated that ACE-2 balances the steady-state levels of both ANGII and ANG1–7 in alveolar epithelial cells (Uhal et al., 2011). Blockade of ACE-2 by a competitive inhibitor DX-600 or siRNA mediated knockdown showed activation of apoptotic markers in AECs (Uhal et al., 2011). ACE-2 limits the accumulation of ANGII and produces ANG1–7 which inhibits JNK phosphorylation and apoptosis induced by either ANGII or by bleomycin. This protective role of ACE-2 was further strengthened by *in vivo* experiments conducted by Imai et al. showing that inhibition or knockout of ACE-2 in mice worsens acid aspiration induced lung injury. Additionally, a recombinant form of ACE-2 protects against acute lung injury in mice (Imai et al., 2005). Moreover, the specific ANG1-7 inhibitory effects were blocked by A779, a receptor antagonist for the Mas receptor which has shown to mediate the anti-apoptotic effects of ANG1-7.

Experimental studies to date noticeably demonstrate the protective actions of ANG1-7/Mas receptor pathway on lung injury through activation of signaling mechanisms (Li et al., 2008; Uhal et al., 2011). Beneficial actions of ANG1-7/Mas include inhibition of tissue remodeling and/or collagen deposition, anti-proliferative and have shown to prevent apoptosis in epithelial cells. These cell type specific signaling pathways downstream of the Mas receptor

demonstrate the importance of ANG1-7/Mas signaling in individual cell types of the lung. Experimental data from our group and others, suggest the potential of ANG1-7, or activators of the Mas receptor, in regulation of apoptosis in AECs.

Although, a significant number of experimental studies elucidated the inhibitory actions of the ANG1-7/Mas pathway in pulmonary cell types and in other cell types, the exact mechanism(s) by which ANG1-7/Mas inhibits JNK phosphorylation and apoptosis in AECs needed to be evaluated. Therefore, it was hypothesized that ANG1-7 constitutively activates a map kinase phosphatase-2 and maintains cell survival. The experimental data herein showed the ability of ANG1-7 to block ANGII-induced apoptotic markers including phospho-JNK, caspase-9, MMP, DNA fragmentation and apoptosis in alveolar epithelial cells. Further, inhibition of ANG1-7 actions by the siRNA mediated knockdown of MKP-2 show the upregulation of MKP-2 by the ANG1-7/Mas pathway. These data support the concept that ANG1-7 upregulates the phosphatase MKP-2 through Mas receptor and thereby maintains low phospho-JNK levels to promote survival in alveolar epithelial cells.

Together, these studies showed the upregulation of MKP-2 by the ANG1-7/Mas pathway that constitutively dephosphorylates JNK and maintains cell survival. Since the beneficial actions of ACE-2 and ANG1-7/Mas are already known, the demonstration of MKP-2 downstream of the ANG1-7/Mas pathway suggests the potential for pharmacological manipulation of AEC apoptosis and related pathogenic conditions in the lung through Mas and MKP-2.

**Role of Angiotensin 1-7/ACE-2 in ER Stress and Hyperoxia**

ER stress induced by physiological conditions or by pharmacological agents cause accumulation of unfolded proteins which in turn activates UPR (Pluquet et al., 2005). Many recent experimental studies have demonstrated that ER stress in AECs can be induced by many harmful agents that lead to AEC apoptosis and lung injury. Therefore, it was theorized that ER stress may induce apoptosis through the autocrine generation of ANGII/ANG1-7 in AECs. To test this hypothesis, mouse lung epithelial cells were challenged with MG132, a synthetic proteasome inhibitor to induce ER stress. The results demonstrate, JNK phosphorylation induced by MG132 was significantly attenuated by the non-selective ANG receptor blocker saralasin and was completely abrogated by ANG1-7. Further, the result suggests a more important role of the ANG1-7 in regulating ER stress-induced apoptosis in lung epithelial cells, relative to the ANGII/ATI receptor interaction. These data show that in human AECs, ER stress-induced JNK phosphorylation is mediated by the peptides ANGII/ANG1-7 and demonstrated effective blockade of pJNK by ANG1-7. These data also may suggest ACE-2/ANG1-7 as therapeutic strategies for ER stress-induced pathogenic lung diseases and might be prevented by manipulation of the ANG system.

Activation of the ANG system was also investigated in lung fibroblasts exposed to hyperoxia. AECs are the main source of ACE-2 in the adult lung. Regulation of cell survival by ACE-2 was investigated by Uhal et al. in AECs demonstrating the knockdown of ACE-2 disrupts the balance between ANGII and ANG1-7 levels in AEC culture media (Uhal et al., 2011). Similarly inhibition of ACE-2, significantly increased ANGII levels in the mouse lung (Li et al., 2008). Additionally, inhibition of ACE-2 by a competitive inhibitor increased caspase-9, an apoptotic

marker in AECs. It was also found that ACE-2 mRNA, immunoreactive protein and enzymatic activity were all high in quiescent mouse and human lung epithelial cells, but were severely downregulated in actively proliferating cells (Uhal et al., 2013a). In support of this theory, downregulation of ACE-2 was observed in lung tissues of experimental mice exposed to bleomycin. This downregulation or loss of ACE-2 observed in the lungs might be due to the high fraction of AECs that are proliferating. Inhibition of ACE-2, induced lung collagen accumulation and in addition, a purified recombinant human ACE-2, attenuated bleomycin-induced lung collagen accumulation in mice (Li et al., 2008). In the recent study conducted, the data show ACE-2 is expressed not only by AECs, but also by fetal human lung fibroblasts. Exposure of human lung fibroblasts to hyperoxia decreased ACE-2 protein levels, but a soluble form of ACE-2 was increased in IMR90 cell culture media. TAPI-2, an inhibitor of ADAM17/TACE significantly reduced the shedding of ACE-2 from the cell surface which shows that hyperoxia with normoxic recovery induces soluble ACE-2 by an ADAM17/TACE sensitive mechanism.

Collectively, data here show the upregulation of the ANG system in ER stress and hyperoxia in lung cells. Further, ACE-2/ANG1-7 might be useful in the management and prevention of hyperoxia or ER stress induced lung injury and may hold the potential as therapeutic strategies for treating pathological conditions in the lung.

## CONCLUSIONS

It is well documented that in many organ systems, signaling mechanisms of the ANGII/ATI pathway are counteracted by the opposing axis ANG1-7/Mas pathway. Only a few studies have explored the downstream signaling factors of the ANG1-7/Mas pathway. However, the different signaling mechanisms of ANG1-7 that were reported could be due to different experimental conditions that were being used in various cell types. The data presented here showed upregulation of MKP-2 by the ANG1-7/Mas pathway and constitutively dephosphorylates pJNK as a cell survival mechanism in lung epithelial cells. However, the molecular mechanism(s) by which ANG1-7/Mas pathway upregulates MKP-2 are currently unknown. It was demonstrated that in vascular smooth muscle cells ANG1-7 releases prostaglandin $I_2$ ($PGI_2$) and increases cAMP levels to reverse ANGII induced phosphorylation of ERK1/ERK2. Further, inhibition of cAMP dependent protein kinase activity prevented the anti-proliferative actions of ANG1-7 (McCollum et al., 2012b). It was also shown that cAMP elevating agents increase MKP-1 in pheochromocytoma (PC-12) cells (Burgun et al., 2000). Similarly, Lara et al. found that activation of a $Na^+$-ATPase in the proximal tubule in response to ANGII was prevented by ANG1-7 through the cAMP/PKA-mediated mechanism and further, ANG1-7 effects were blocked by the Mas receptor antagonist. Moreover, it was found that ANG1–7 prevented ANGII-induced activation of $Na^+$-ATPase through a PKA-mediated mechanism that reversed elevated levels of PKC activity (Lara et al., 2010). In an earlier publication, it was shown that ANGII activates PKC and further, blockade of PKC prevented AEC apoptosis (Uhal et al., 2011). Thus, it will be of high interest to determine whether cAMP/PKA pathway is involved in upregulation of MKP-2 in AECs and whether siRNA mediated silencing of cAMP-dependent

protein kinase could reverse the ability of ANG1-7 to block ANGII induced effects. Likewise, it would also be interesting to investigate whether cAMP analogues could prevent ANGII induced JNK phosphorylation and AEC apoptosis. Further, to investigate other signaling pathways by which ANG1-7/Mas inhibits AEC apoptosis, signaling arrays can be used to test the activation of genes in control vs. treated groups. Although, multiple signaling pathways might be involved in downstream of ANG1-7/Mas pathway, the data presented here demonstrate a key role of MAPK/MKP signaling and suggests the potential for pharmacological manipulation of pathogenic conditions in the lung through Mas and MKP-2.

The data here also demonstrated that manipulation of the ANG system can rescue lung cells in response to ER stress or hyperoxia. The exact mechanism(s) by which ER stress activates the ANG system are not understood clearly. But it is highly likely that both axes of the autocrine ANG system are involved in regulating ER stress induced apoptosis of AECs. Moreover, previous work showed a significant loss or downregulation of ACE-2 in response to apoptotic inducers through mechanisms that are yet to be elucidated. Further, identifying the key signaling mechanisms that upregulate ADAM17/TACE in hyperoxia/ER stress induced lung injury will be an interesting topic to investigate. Collectively, these data suggest the importance of regulation of AEC apoptosis by manipulating the autocrine ANG system in lung cells and may provide therapeutic strategies to prevent lung diseases that involve AEC apoptosis.

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