Partnership between the aryl hydrocarbon receptor (AHR) and RELB regulates cigarette smoke-induced cyclooxygenase-2 (COX-2) expression

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List of Abbreviations

Abbreviation	Meaning
AHR	Aryl hydrocarbon receptor
ARE	AU rich element
ARNT	AHR nuclear transporter
B[*a*]P	Benzo[*a*]pyrene
BSA	Bovine serum albumin
CF	Cystic Fibrosis
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRE	Cyclic adenosine mono-phosphate response element
CRM	Chromosome maintenance region
CSE	Cigarette smoke extract
Cyp	Cytochrome p450
DMSO	Dimethyl sulphoxide
DRE	Dioxin response element
ECM	Extracellular Matrix
FEV_1	Forced Expiratory Volume in one second
FICZ	6-formylindolo[3,2-b]carbazole
FVC	Forced Vital Capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HSP90	Heat shock protein 90
HUR	Human Antigen R
IFNγ	Interferon gamma
IGEPAL	Octylphenoxypolyethoxyethanol
IKKα	Inhibitor of kappa beta kinase
IL	Interleukin
IPF	Idiopathic lung fibrosis
ITE	2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	mitogen activated protein kinase
MCP	Macrophage chemoattractant protein
miR-146a	micro RNA 146a
miRNA	microRNA
MMP	Matrix Metalloproteinase
mPGES	Microsomal prostaglandin endoperoxidase synthase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NF-κβ	Nuclear factor kappa beta
NIK	NF-κβ inducible kinase
PBS	Phosphate buffered saline
PG	Prostaglandin (D,E, F,H,I)
PLA_2	Phospholipase A two
RBP	RNA binding protein
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
sAHRm	Selective AHR modulator

siRNA	Small interfering RNA
SOD	Superoxide dismutase
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TNFα	Tumour necrosis factor alpha
TRAF	TNFα receptor-associated factor
TTP	Tristetraprolin
TXA_2	Thromboxane
UTR	Untranslated region
XAP2	X-associated protein 2
XRE	Xenobiotic response element

Partnership between the aryl hydrocarbon receptor (AHR) and RELB regulates cigarette smoke-induced cyclooxygenase-2 (COX-2) expression

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease of the lungs caused by cigarette smoke exposure; COPD is also now the third leading cause of death worldwide. Controlling lung inflammation is a priority in COPD patients, but currently-available medications offer little relief. Thus, new therapeutic targets represent a major unmet need. Previously, the aryl hydrocarbon receptor (AHR) has been shown to suppress cigarette smoke-induced inflammation. The AHR is a ligand-activated transcription factor, and well-established for its response to xenobiotic ligands. AHR-mediated suppression of smoke-induced inflammation requires the NF-κβ family member RELB. To date, nothing is known about the expression of AHR or RELB in subjects with COPD. Therefore, we investigated the expression of the AHR and RELB in human lung fibroblasts derived from Control (Non-smoker), Smoker and COPD subjects, as well as the mechanism through which they repress the production of the inflammatory protein cycloxygenase-2 (COX-2) in response to cigarette smoke extract (CSE). There was reduced AHR expression in COPD subjects, which was associated with increased expression of COX-2 protein (but not mRNA) in quiescent COPD fibroblasts. Inhibition of AHR activity in lung fibroblasts by the pharmacological AHR antagonist CH-223191 increased COX-2 protein induction by CSE. Mechanistically, this increase in COX-2 protein corresponded with increased cytoplasmic shuttling of the RNA binding protein HUR which is known to prolong the half-life of RNA transcripts - including COX-2. HUR was observed to be cytoplasmically-localized in lung tissue from COPD subjects, but not Control, suggesting altered regulation of HUR in COPD subjects. Another protein

associated with the suppressive function of the AHR- RElB, was reduced in both Smoker and COPD lung fibroblasts, suggesting cigarette smoke may contribute to the reduced expression. siRNA-knockdown of RELB increased the production of Cox-2 mRNA in response to CSE or IL-1β, supporting that RELB contributes to the suppression of Cox-2. We hypothesized this may be due to miR-146a, an anti-inflammatory microRNA (miRNA) which targets Cox-2 mRNA for degradation. miR-146a basal expression was not significantly different between the subject groups. However, only Control fibroblasts increased miR-146a expression in response to CSE. siRNA knockdown of RELB abrogated the expression of miR-146a in response to IL-1β, but not CSE, suggesting RELB repression of Cox-2 mRNA does not involve miR-146a, but that RELB may regulate miR-146a under certain stimuli. Collectively, these data support the regulatory role of AHR and RELB in cigarette smoke-induced inflammation, and thus represent promising new cellular targets with the potential for controlling inflammation characteristic of COPD.

La maladie pulmonaire obstructive chronique (MPOC) est une maladie inflammatoire chronique des poumons causée par l'exposition à la fumée de cigarette, maintenant au troisième rang des causes de mortalité mondiaux. Le contrôle de l'inflammation pulmonaire est hautement prioritaire pour les patients atteints de MPOC, mais les médicaments actuellement disponibles ne réduisent guère cette inflammation. Les nouvelles cibles thérapeutiques restent donc un important besoin non satisfait. La RAH est bien établi comme une réponse à des ligands xénobiotiques toxiques. La suppression par l'entremise de RAH de l'inflammation causée par la fumée de cigarette nécessite un membre de la famille NFκβ. Jusqu'à date, rien n'est connu de l'expression de RAH ou

RELB parmi les patients atteints de MPOC. Par conséquent nous avons examiné ci-dessus la relation entre l'expression de RAH et RELB dans les fibroblastes de poumon humains dérivés des sujets témoins (non-fumeurs), fumeurs, et sujets atteints de MPOC, ainsi que le mécanisme par lequel ils répriment la production de la protéine inflammatoire cyclo-oxygénase 2 (COX-2) en réponse à l'extrait de la fumée de cigarette (EFC). L'expression des protéines RAH a été réduite parmi les fibroblastes des sujets atteints de MPOC, qui était aussi associée avec une expression accrue de la protéine COX-2 (mais pas ARNm) parmi les fibroblastes quiescents. L'inhibition de l'activité de RAH dans les fibroblastes pulmonaires par l'antagoniste pharmacologique CH-223191 a augmenté l'induction de COX-2 par EFC. Mécaniquement, cette augmentation de la protéine COX-2 a correspondu avec un accroissement du transport cytoplasmique de la protéine de liaison d'ARN HUR, qui est connu pour prolonger la demi-vie des transcrits d'ARN, y compris COX-2. Il a été observé que HUR est cytoplasmiquement localisé dans le tissu pulmonaire des sujets atteints de MPOC, mais non pas les témoins, suggérant une régulation altérée de HUR parmi les sujets atteints de MPOC. Une autre protéine associée avec cette fonction de suppression de la RAH=RElB a été réduite parmi les fibroblastes pulmonaires des sujets-fumeurs et sujets atteints de COP, suggérant que la fumée de cigarette puisse contribuer à cette expression réduite. pARNi (ou siRNA) anéantissement de RELB accroissait la production de COX-2 ARNm en réponse à CSE ou IL-1, appuyant l'observation que RELB contribue à la suppression de COX-2. Nous avons formulé l'hypothèse que cela pourrait être du à miR-146a, un micro-ARN (miARN) qui cible COX-2 mRNA pour dégradation. L'expression basale de miR-146a ne fut pas significativement différente parmi les divers groupes de sujets. Pourtant seulement les

fibroblastes des sujets témoins ont accru l'expression de miR-146 en réponse à l'EFC. pARNi (ou siRNA) anéantissement de RELB a abrogé l'expression de miR-146 en réponse à IL-1β, mais pas l'EFC, suggérant que la répression par RELB de COX-2 ARNm n'entraîne pas miR-146a, mais que RELB pourrait réglementer miR-146a soumis à certains stimuli. Collectivement, ces données étayent le rôle régulateur de RAH et RELB dans l'inflammation induite par la fumée de cigarette, et donc représentent de nouvelles cibles cellulaires prometteuses, pleines de potentiel de contrôler l'inflammation caractéristique de MPOC.

Chapter 1- Introduction

Chronic Obstructive Pulmonary Disease

1.1 An overview of COPD

Chronic obstructive pulmonary disease (COPD) is primarily caused by inhalation of cigarette smoke or biomass fuels[1,2], and is a worldwide health concern. The 2010 Global Burden of Disease Study reports that COPD is the third highest cause of worldwide mortality[3], an increase from the fourth leading cause of death since 1990[4]. This upward trend is not likely to change in the near future, as COPD mortality is expected to increase in the next 15 years[4–6]. Currently, COPD is considered an under-reported disease with an estimated prevalence of 4.4% (or approximately 700,000 Canadians)[5]. As a progressive and chronic disease, COPD negatively impacts a patients' quality of life for many years, while decreasing the number of years of life remaining[7]. Furthermore, COPD increases the risk of other diseases, including cardiovascular diseases and heart failure[8–10]. Complex interventions involving education or exercise training can decrease the burden on the healthcare system, and have been shown to be effective at reducing mortality and the cost of hospitalization[11], which is nearly 4 billion dollars in Canada annually[12]. Collectively these projections highlight the need for strategies to combat the rate at which COPD is increasing.

1.2 Characteristics and Symptoms of COPD

According to the GOLD standard (Global Initiative for Chronic Obstructive Lung Disease) which is widely used in many countries including the United States, Canada and

UK, COPD is diagnosed by assessing the degree of airflow limitation via spirometry[6]. To do this, the FEV_1, or the 'forced expiratory volume in 1 second', is measured and compared to the FVC, or 'forced vital capacity'. Essentially, the percentage of total breathing volume a subject can exhale in one second is measured, and compared to average values, normalized for age, weight and height[6,13,14]. Patients exhibiting a FEV_1/FVC ratio of less than 80% of predicted are diagnosed with COPD. Greater airflow limitation indicates increased severity of COPD, which is divided into 4 stages: GOLD I: mild (≥80%), GOLD II: moderate (80≥50%), GOLD III: severe (50≥30%) and GOLD IV: very severe (<30%). Lung function is measured before and after administration of a bronchodilator, such as a β-agonist, to assess the degree to which airflow limitation is reversible. This helps clinicians distinguish COPD from asthma, as COPD airflow limitation is not fully reversible by bronchodilators. Chronic bronchitis, or the production of sputum for at least three months in two consecutive years[2,6], is also a sign of COPD progression. Patients with COPD may experience symptoms of dyspnea or breathlessness and may be accompanied by cough. These symptoms are often mistaken for a virus or 'cold' at early stages, and must worsen to the point at which patients seek treatment in order to diagnose COPD. Unfortunately, COPD is under-diagnosed because symptoms often go undetected for many years as the body compensates for the gradual progression of emphysema[6,15]. Emphysema is a general term that describes the destruction of lung tissue, particularly the alveolar sacs required for normal gas exchange. Emphysematous destruction of tissue decreases the elastic recoil of the lungs, and impairs the lungs ability to forcibly exhale. Healthy subjects are able to expel at least 80% of the air in their lungs in a short expiratory effort, while COPD subjects may expel as little as 30% in severe

cases[6,16]. This is called expiratory flow limitation, and is a major source of the dyspnea[17]. The body compensates for expiratory flow limitation by increasing resting lung volumes, which leads to hyperinflation of the lung[18], or 'air trapping'. Hyperinflation of the lung assists expiration by taking advantage of increased elastic force at larger lung volumes, but does so at the cost of decreased inspiratory capacity[19,20]. Symptoms of dyspnea and cough worsen with COPD severity, whereupon chest tightness, fatigue or listlessness as a result of increased breathing effort are reported[6,12].

At the cellular level, COPD is characterized by physiological changes such as destruction of alveolar sacs (emphysema), narrowing of the small airways and enhanced production of inflammatory mediators[5,6,21]. Cessation of smoking is still the single greatest intervention to halt the progression of COPD[22], but it cannot undo the tissue destruction already present. Halting inflammation is a major target for the treatment and management of COPD through anti-inflammatory medications such as β-agonists, such as Ventolin® and Atrovent®, and inhaled glucocorticoids such as budesonide[6,23–25]. These medications have been met with varied success[23,26] and corticosteroids can exacerbate the already increased risk of viral and bacterial infection in COPD that can further increase inflammation[27–29]. This highlights the need for new strategies to deal with inflammation in COPD.

1.3 Inflammation and COPD

1.3.1 Overview of Inflammation in COPD: Inflammation is a necessary part of the immune response to injury or pathogen infiltration of the body[30,31]. As a general response

that is non-specific to the source of injury or infection, acute inflammation is generally associated with the innate immune system. Inflammatory responses are generally initiated by cells in affected tissues[32,33], followed quickly by specific innate, then adaptive immune cells through the production of inflammatory mediators such as cytokines and chemokines. Prolonged or chronic inflammation creates an inflammatory environment that can dramatically remodel affected tissues[34].

As previously stated, cigarette smoke is the most frequent cause of inflammation associated with COPD[1,2]. Cigarette smoke is a highly heterogeneous mix of greater than 5000 compounds[35,36], and a full description is beyond the scope of this work, however multiple pro-inflammatory agonists have been identified. These pro-inflammatory mediators including acrolein, benzo[*a*]pyrene (an Aryl hydrocarbon receptor agonist) and activators of oxidative stress like nitrogen oxide[35–37]. Data also indicates that high concentrations of carbon dioxide may also contribute to infammation[38].

Lung inflammation caused by cigarette smoke, and characteristic of COPD, is highly neutrophillic[2,33,39], but also includes inflammatory cells such as alveolar macrophages[2] and T-lymphocytes[28,40]. Continual exposure to cigarette smoke promotes CD8+ T-lymphocyte recruitment to the lung[28]. Interestingly, smoking status and number of cigarettes smoked has been shown to correlate best with CD8+ T-lymphocyte recruitment[41], rather than disease status. CD8+ T-lymphocytes are recruited in response to smoke, in healthy and COPD subjects. Recruited CD8+ cells are strongly activated in the bronchial mucosa of COPD patients[40], and may also contribute to alveolar destruction and neutrophil recruitment. Recruited cells produce numerous mediators, including cytokines, chemokines, lipid products, and matrix degrading enzymes. Production of

neutrophil chemoattractants like IL-8 causes neutrophils infiltration to the lung[2,39]. Production of both IL-1β and TNFα[2,33] is increased, with subsequent activation of macrophages, contributing to the production of IL-6[2] and matrix metalloproteinases (MMP) such as MMP-9 or MMP-12[2,42]. Production of MMPs is augmented by neutrophil elastase, which is a major paradigm for the progression of emphysematous COPD due to the degradation of elastin and loss of lung elasticity[27,43]. Interestingly, cigarette smoke is also associated with decreased expression of anti-proteases like α_1-antitrypsin[43–45], and smoke has been shown to prevent α_1-antitrypsin from binding to elastin[46,47]. Cigarette smoke therefore increases the production of multiple inflammatory intermediaries and enables neutrophil elastase to destroy connective tissue leading to destruction of the small airways of the lungs[1,48,49], exacerbating COPD. In addition to classic immune cells, it is now recognized that structural cells, including epithelial and endothelial cells[2] as well as fibroblasts[2,50,51] are important sources of inflammatory signals found in COPD.

1.3.2 Pulmonary Fibroblasts:

The primary role the fibroblast is to synthesize and maintain extracellular matrix (ECM), providing structure and elasticity to the lung. Fibroblasts are the most numerous cell type in connective tissues[52] making up roughly 25% of the lung dry weight[53]. Fibroblasts, including lung fibroblasts, can be recognized by their spindle-shaped morphology[54] and abundant rough endoplasmic reticulum, necessary for their prolific production of ECM[52]. Characterization of fibroblasts relies on the expression the intermediate filament vimentin, but the absence of desmin, α-smooth muscle actin, and keratin[54]. This designates fibroblasts as cells from a mesenchymal linage, and distinguishes them from skeletal muscle cells, myofibroblasts and epithelial cells. As the

most important producers of ECM, fibroblasts secrete collagen type I and type III reticular fibers that support the structure of the ECM[55]. Fibroblasts also produce non-collagenous matrix proteins such as proteoglycans and elastin[56]. Proteoglycans are negatively charged due to heavy glycosylation that contains sulphate groups. Due to this negative charge, proteoglycans attract cations which regulates the osmotic pressure in connective tissue interstitial fluid[57]. Within the lung, interstitial osmolarity may also affect the ability of the lung to collapse and expand via stabilization of the network of collagen and elastin fibers[58]. Elastin fibers give connective tissues their elasticity and is of paramount importance for elastic recoil of the lung[55,59]. Progressive degradation of elastin in COPD patient lungs reduces expiratory force due to the loss of lung elasticity[60], and some studies have suggested elastin fragments as putative biomarkers for emphysematic changes in the lung[60,61].

Fibroblasts are also involved in the process of wound healing after tissue injury. Tissue injury in the lung encompasses physical trauma, environmental damage (such as cigarette smoke), infectious agents or inflammatory damage from pathology and may involve the formation of scar tissue due to ECM deposition from fibroblasts[62]. Thus, fibroblasts are involved that fibrotic diseases such as cystic fibrosis (CF)[63], idiopathic lung fibrosis (IPF)[21,64] and Cirrhosis[65]. Fibrosis is particularly problematic in the lung, as it stiffens the tissue, reducing elasticity and increasing the effort of breathing[66]. This is because many fibroblasts exist within the alveoli-containing respiratory tissues of the lung, or parenchyma. Fibrotic scarring in and around alveoli decreases or prevents gas exchange due to air sac thickening[21,66,67]. In addition to these well-characterized

functions, fibroblasts are also important contributors of lung inflammation associated with cigarette smoke and the development of COPD.

1.3.3 Fibroblasts in inflammation associated with COPD: Fibroblasts are now known to be important contributors to inflammation, particularly chronic inflammation[31,68]. Numerous studies show that fibroblasts respond to inflammatory mediators such as IL-1β and TNFα, and rapidly produce an array of cytokines, chemokines, lipid mediators and proteases[31,32,69]. Fibroblasts are also a strong source of not only inflammatory mediators, but also MMPs. In short, it has been suggested fibroblasts are responsible for sustaining the inflammation in COPD[51,70]. This is supported by observations that fibroblasts derived from patients with COPD secrete more IL-6, IL-8, MMP-9 and PGE_2 which collectively contribute to a persistent pro-inflammatory environment[2,70,71]. In this way fibroblast production of IL-6 and IL-8 may facilitate chronic inflammation by promoting the infiltration and survival immune cells. Continued production of IL-8 is necessary for persistent neutrophil recruitment to the lungs, as COPD is associated with increased neutrophil recruitement[72]. MMPs are critical for normal maintenance of the ECM, but over-expression of MMP-1, MMP-2, MMP-9, and MMP-12 in COPD[73,74] by fibroblasts also contribute to inflammation. Degradation of the ECM by MMP-2 and -9, for example, facilitates infiltration of the lung by inflammatory cells[74,75], while MMP-12 may be elastolytic, and contribute to the degradation of lung elastin[61]. IL-6 from fibroblasts promotes the differentiation and survival of CD8+ T cells which infiltrate the lung in response to cigarette smoke[41,76,77], while also increasing the production of cycloxygenase-2 (COX-2)[76], an inducible enzyme whose expression is increased in COPD.

1.4 Cycloxygenases in COPD

The COX isoforms COX-1 and COX-2 (also known prostaglandin-endoperoxide synthase, or PTGS) are responsible for the production of prostaglandins, and thromboxane (TXA_2)[78]. COX-1 is responsible for basal production of arachidonic acid metabolites and is constitutively expressed[78], whereas COX-2 is the inducible form of the enzyme that is upregulated upon exposure to inflammatory stimuli such as IL-1β or cigarette smoke[42,79]. Arachidonic acid derived from the lipid membrane by phospholipase-A_2 (PLA_2) or diacylglycerol lipase can be used as a substrate by either of the COX isoforms to produce prostaglandin H_2 (PGH_2) [78]. Production of PGH_2 by COX-1 or COX-2 is the rate-limiting step in the production of other prostaglandins[80]. PGH_2 can be converted to PGE_2, PGI_2 (prostacyclin), PGD_2, PGF_2, or TXA_2 by specific isomerases[81,82]. PGJ_2 is an additional prostaglandin may be produced via the secondary metabolite PGD_2[82]. Collectively, PGs modulate cytokine and inflammatory mediator production in a tissue and signal specific manner through the regulation of COX expression[39,78,82,83].

Aberrant COX-2 expression is associated with numerous diseases[79,84]. Increased COX-2 expression is implicated in the pathology of cancers of the breast, pituitary and colon[85–88], as well as the inflammation characteristic of COPD[39,89].. Both TXA_2 and PGE_2 are elevated *in vitro* or in breath condensate[90–93]. The synthase response for the terminal production of PGE_2-mPGES-1- is also increased in COPD, alongside COX-2[94]. PGE_2 can stimulate many inflammatory mediators present in COPD, such as IL-1β, TNFα, MCP-1, IL-6 and IL-8[2,85,95,96]. Pharmacological inhibition of COX-2 is a successful strategy for

halting the production of excessive PGE_2[97–99]. Inhibition of COX-2 mediated PGE_2 production is well known to decrease the risk of cancer[87,99] and may provide protection against emphysema[100]. Thus, research has focused on pathways regulating inflammation, including COX-2 production as potential therapies.

1.5 *Regulation of Cox-2*

Cox-2 is regulated at several levels, including both the transcriptional and post-transcriptional[101,102]. Transcription of *Cox-2* mRNA is controlled through the transcriptional elements within the *Cox-2* promoter. These transcriptional elements include, but are not limited to, two κβ sequences, two cyclic AMP response elements (CRE) and a nuclear factor for IL-6 response (NF-IL6)[102] which can be bound by transcription factors. As such, *Cox-2* expression is highly responsive to early inflammatory stimuli such as IL-1β or IL-6[71,103]. Many of these stimuli, such as IL-1β and TNFα, induce *Cox-2* via the NF-κβ transcription factor[104,105]. *Cox*-2 regulation has been well-studied at the transcriptional level in fibroblasts, epithelial cells and macrophages[102]. In addition to being studied by cell type, cigarette smoke has been shown to enhance *Cox-2* expression[39,89,106]. Recent pooled genome wide association studies have also shown increased *Cox-2* expression, in addition to altered expression of many other genes, is a common consequence of smoking[107–109]. These studies demonstrate how elevated *Cox*-2 is common amongst smokers and COPD patients, in addition to increases in transcription factors like NF-κβ that regulate *Cox-2*. Interestingly, when genome wide expression from smokers, non-smokers, and former smokers by

cessation time were compared, it demonstrated how cessation of smoking returns some (not all) genes to a pre-smoking expression pattern, while others remained permanently altered[107].

Cox-2 is also regulated at the post-transcriptional level. Post-transcriptional regulation of *Cox-2* is typified by alterations in mRNA stability or protein translation, and occurs through factors such as RNA binding proteins (RBPs) and microRNAs (miRNAs)[93,101,103,110]. Both RNA binding proteins and miRNAs are known to influence the level of COX-2 expression via regulation of *Cox-2* mRNA stability. The *Cox-2* mRNA transcript, like many RNA trancripts, contains a region within the 3' UTR that is rich in adenine and uracil, called the AU rich element (ARE). The ARE regulates the decay of mRNAs and can be bound by RBPs that increase or decrease mRNA stability[111,112]. Human antigen R (HUR) is a well-known example of an RBP that is capable of stabilizing RNA transcripts[101]. When HUR binds the ARE of the *Cox-2* mRNA transcript, the transcript is stabilized against degradation, prolonging its half life in the cytoplasm[112,113], thus increasing the amount of translated COX-2. Conversely, miRNA are non-coding mRNAs, twenty two-nucleotides in length, that target specific mRNA transcripts for degradation or translational inhibition based on the degree of complementarity[114]. miRNAs that match the mRNA sequence perfectly will initiate degradation as part of the RNA induced silencing complex (RISC)[114,115]. Imperfect complementary sequences between miRNAs and mRNA transcripts results in translational inhibition[114]. Some miRNAs such as miR-146a are known to target *Cox-2* transcripts. [93,116,117] and has previously been associated with COPD[93]

Thus, the suppression of COX-2 expression in COPD requires control at both the transcriptional and post-transcription levels. One protein that may contribute to both facets of COX-2 regulation is the aryl hydrocarbon receptor (AHR).

The Aryl Hydrocarbon Receptor (AHR)

1.6.1 Classical role of the AHR:

Since its discovery in 1976[118], the AHR has become a well-established regulator of the response to toxicological compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or benzo[*a*]pyrene (B[*a*]P). The AHR is held in a cytoplasmic complex containing dual copies of heat shock protein 90 (HSP90), X-associated protein 2 (XAP2)[119,120]. A more recently discovered member of the complex, p23, has been suggested to protect the AHR from proteolytic degradation[121]. Upon ligand binding, the AhR undergoes a conformational change that exposes a nuclear localization signal[122]. Nuclear import of AHR may occur via importin-β and mediated by XAP2 and HSP90[122]. Once in the nucleus the AHR forms a heterodimer with the AHR nuclear transporter (ARNT)[120,123]. This AHR-ARNT heterodimer binds to DNA consensus sequences called xenobiotic response elements (XREs), also called dioxin response elements (DREs). XREs contain a variant of the consensus sequence 5'-T/GNGCGTGA/CG/CA-3'[124] and are found within the promoter of detoxifying genes which include the Cytochrome P450 family (i.e.*Cyp1a1* or *Cyp1b1*)[123,125]. The AHR recruits initiating transcription factors like p-TEFb and enables the phosphorylation of RNA polymerase II to initiate

transcription[126]. Figure 1.1 depicts the classic AHR pathway in response to xenobiotic ligation.

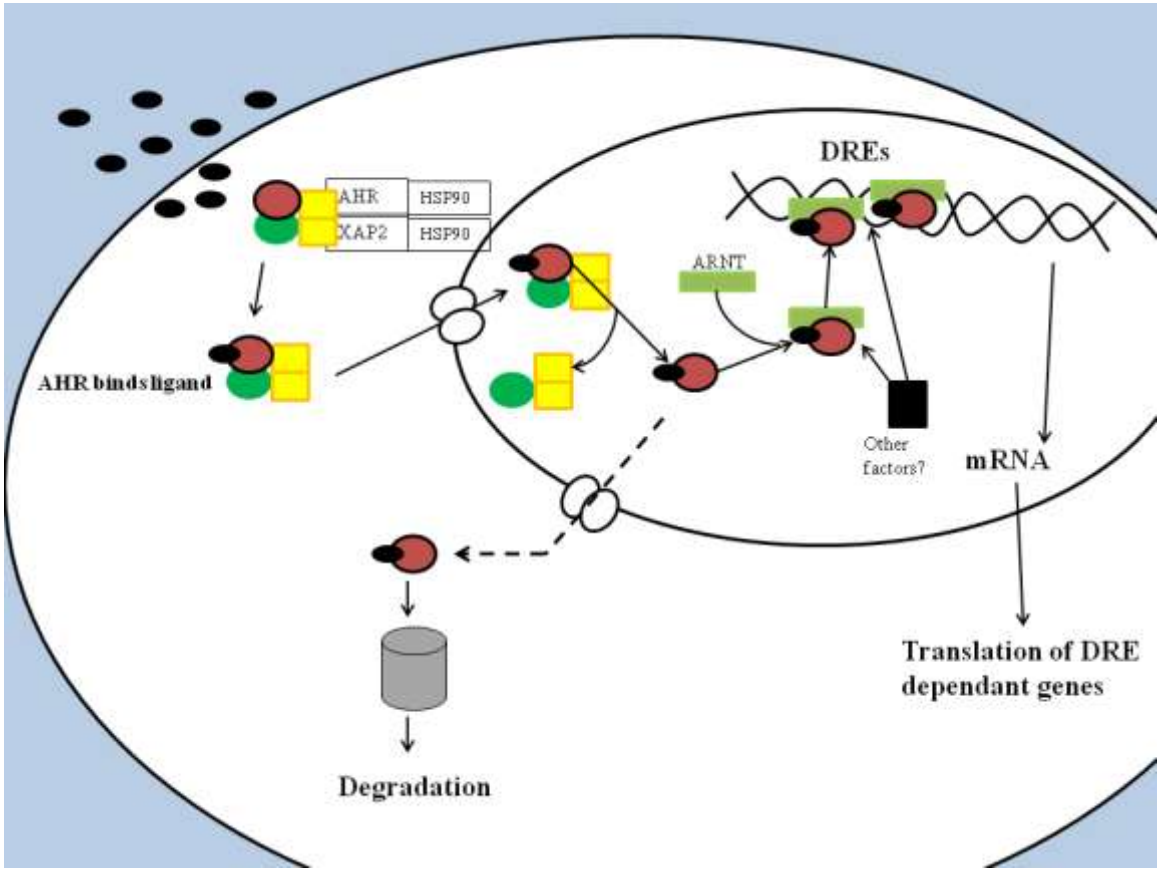

Figure 1.1 The AHR pathway, as activated by a classic ligand like dixoin, results in the upregulation of DRE-dependent genes such as *Cyp1b1*.

1.6.2 AHR expression:

Although the AHR is ubiquitously-expressed, certain tissues express the AHR at higher levels compared to others. The liver, lungs and kidneys of rats (all tissues associated with detoxification or high environmental interaction) express high amounts of the AHR[127]. This pattern tissue specific expression of the AHR is consistent across many species, including humans[127,128]. Whether the AHR is altered in inflammatory lung diseases such as COPD is not known.AHR expression appears to be critical for proper development, as although AHR knockout is not embryonically lethal, only 5% of an expected 25% of AHR null mice survive beyond a week[129]. AHR-null mice exhibit hepatic and renal fibrosis and smaller livers than wildtype mice[130,131]. Furthermore, the lungs of AHR-null mice are susceptible to alveolar simplification, or reduced number of alveolar sacs and splenomegaly[129,132]. In addition to development, AHR-null mice also exhibit immunopathology, with increased neutrophil infiltration in the lung, and reduced periarterial lymphoid sheaths in the spleen[133,134]. Collectively, these developmental changes due to knockout of the AHR point to a physiological role beyond regulation of xenobiotic responses.

1.6.3 The emerging physiological role of aryl hydrocarbon receptor:

Despite the identification of several putative endogenous ligands such as 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) or 6-formylindolo[3,2-b]carbazole (FICZ)[135–137], the physiological role of the AHR remains a mystery. ITE was isolated from lung tissue and stimulates transcription of cytochromes via the classical AHR pathway[135]. FICZ was identified as a metabolite of tryptophan created via exposure to UV radiation[136], and has been shown to promote the differentiation of regulatory T cells[71,138]. Additional studies have reported the existence of selective AHR modulators or SAHRMs. SAHRMs bind the AHR but do not induce AHR-dependant gene transcription, and instead reduce expression of inflammatory mediators without the transcription of classic XRE genes (*Cyp1a1, Cyp1b1*)[139]. Potential AHR modulation by SAHRMs, as well as the fact that recently discovered endogenous ligands ITE and FICZ do not exhibit the toxicity of traditional AHR ligands (*e.g.* TCDD), makes the AHR useful from the standpoint of potential anti-inflammatory treatments[136,138]. This is also supported by recent studies that AHR loss results in enhanced sensitivity to environmental irritants and production of inflammatory mediators in immune cells, such as T cells and B cells[140,141], or structural cell types such as fibroblasts[106,142].

Much of what we know about the AHR as a regulator of inflammation comes from recent studies demonstrating the AHR suppressing inflammation due to cigarette smoke exposure. For example, $AHR^{-/-}$ mouse lung fibroblasts challenged with CSE express more COX-2[142]. Similarly, studies utilizing AHR-deficient mice exposure to cigarette smoke also have more pulmonary inflammation, including levels of PGE_2. Collectively, these studies show the AHR is necessary to inhibit production of COX-2

and PGE_2 in both *in vivo* and *in vitro* in response to cigarette smoke[89,106]. Interestingly, the mechanism by which the AHR represses the expression of COX-2 in response to cigarette smoke occurred via an understudied member of the NF-κβ family known as RELB[142].

RELB - The non canonical NF-κβ pathway

1.7 RELB is a repressor of inflammation

The NF-κβ family of proteins is one of the most important sets of transcription factors regulating inflammation. Genes such as *Cox-2*, *IL-2, IL-8,* and *MCP-1* are examples of at least 400 genes regulated by NF-κβ activation[143]. The NF-κβ family of proteins consists of five members- NF-κβ1, NF-κβ2, c-REL, RELA, and RELB[144]- all of which share a common REL homology domain[143]. These proteins form heterodimers in the cytoplasm, and is casually referred to as the NF-κβ transcription factor. This is typically a heterodimer of RELA and a proteolytically processed form of NF-κβ1 (p105) called p50[145,146]. This pathway of RELA/p50 heterodimers is termed the classic or canonical NF-κβ pathway (Figure 1.2). Strong inflammatory signals that robustly increase gene expression, such as TNF-α or IL-1β, initiate nuclear translocation of RELA:p50 within 30 minutes[147], allowing RELA:p50 to regulate target genes via binding to κβ sites of target genes. RELA:p50 translocation primarily increases gene expression of a battery of inflammatory genes such as IL-8, IL-6, and COX-2[148]. In contrast to the

classical or canonical pathway, the non-canonical pathway involving NF-κβ member RELB is primarily seen as repressor of inflammation (Figure 1.2). Activation of the non-canonical NF-κβ pathway is regulated by the NF-κβ inducible kinase (NIK)[149,150]. Continuous synthesis of cytoplasmic NIK regulated via a system of constant ubiquitination and proteasomal degradation. In the absence of an activating signal, TRAF2 and TRAF3 recruit an E3-ubiquitin ligase to associate with NIK and initiate degradation. In the presence of an inflammatory signal such as TNFα, TRAF2 and 3 dissociate from NIK, and expression of NIK is allowed to continue[151]. This can also be initiated by more selective agonists of the non-canonical pathway such as CD40 ligand or Lymphotoxin-β, TNFα family members[149,152]. Accumulation of NIK to initiate the signal cascade requires time for protein synthesis, and typical non-canonical activation is seen after 2-3 hours[153].

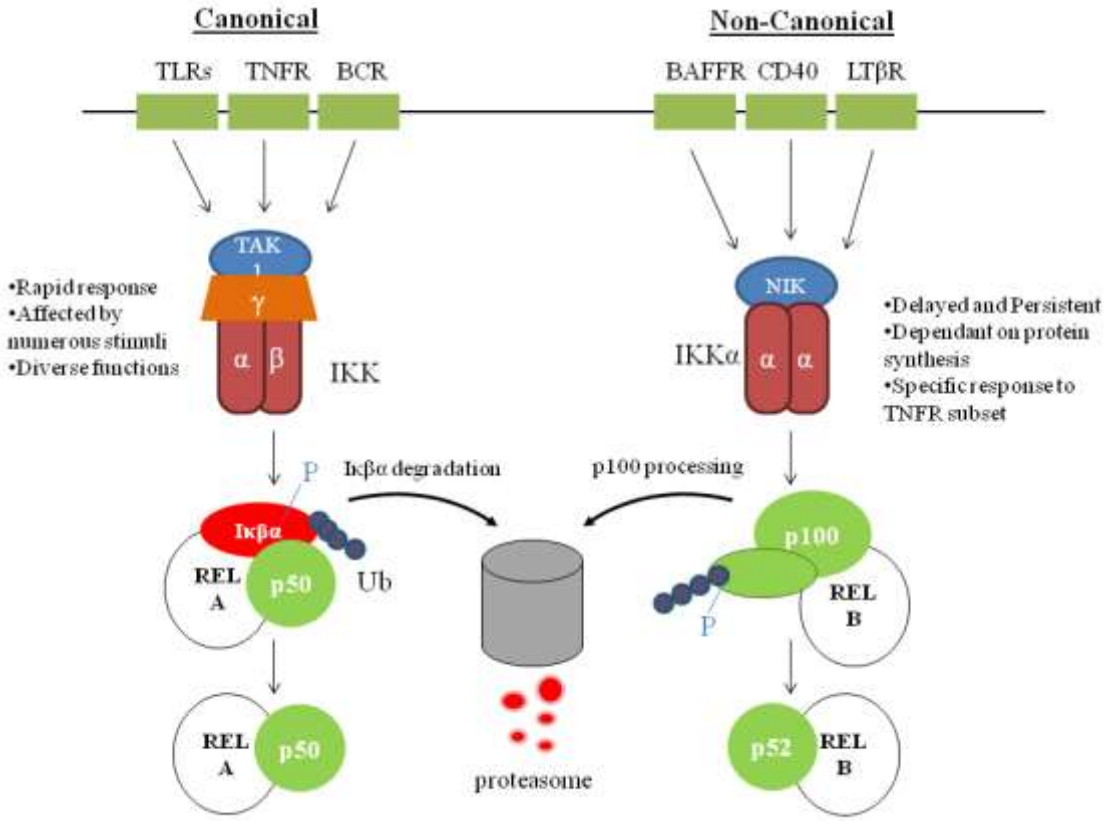

Figure 1.2 Activation of both the Canonical and the Non-Canonical NF-κβ pathways. Activating receptors initiate the corresponding signal cascade, resulting in proteolytic processing and translocation of RELA or RELB-containing heterodimers.

Once accumulated, NIK triggers a cascade of phosporylation in which it phosphorylates dimers of the inhibitor of kappa beta kinase (IKKα), that go on to phosphorylate NF-κβ2. NF-κβ is also known as p100 and is the regulatory subunit of the non-canonical NF-κβ dimer: p100:RELB[143,149]. Phosphorylation of p100 triggers its ubiquitination and it is proteolytically cleaved, forming active p52. RELB:p52 dimers translocate to the nucleus, whereupon they can bind κβ sites and repress gene expression. The mechanism by which RELB represses genes is not well understood, as RELB:p52 complexes are known to repress inflammatory genes like TNFα[106] or COX-2[106], but activate other genes such as superoxide dismutase (SOD)[154,155]. RELB:p52 complexes are known to bind κβ promoter sites and may compete for binding with the canonical pathway[149,156]. The notion of RELB as a repressor of inflammation is strengthened by the excess inflammation seen in $RELB^{-/-}$ mice[157]. These mice exhibit widespread inflammation and profound infiltration of lymphoid cells, particularly in the liver, and lungs. Extramedullary hematopesis, characterized by increased erythroid cells production was found in the spleen and liver. Furthermore, these mice are hypersensitive to inflammatory stimuli and challenge resulted in increased levels of IL-1β, TNFα, IL-2, and IFNγ, as compared to wildtype animals[157]. RELB over-expression also suppressed cigarette smoke-induced lung inflammation, highlighting the anti-inflammatory actions of RELB in the respiratory system[158].

It is now known that RELB and the AHR are physically and mechanistically associated. AHR-deficient mice exhibit heightened inflammation in response to both endotoxin[106,142] and cigarette smoke[106,142], with concomitant loss of RELB expression. Vogel et al. demonstrated a physical link between AHR and RELB within the nucleus in

U937 cells[159]. These AHR:RELB dimers were capable of binding κβ sites, XREs, as well as a previously unrecognized AHR:RELB sequence. However, the link between RELB and the AHR and remains largely unexplored, especially in the case of cigarette smoke-induced diseases such as COPD.

Aims of the Present Study

Cigarette smoke is the most important risk factor for COPD, a chronic obstructive lung disease with high morbidity and mortality. Cigarette smoke contributes to the inflammatory component of COPD, which is characterized in part by the heightened expression of COX-2 protein. The control of COX-2 in response to cigarette smoke occurs at multiple levels and is potently suppressed by expression of the AHR. Whether AHR expression is altered COPD, and thus contributes to heightened inflammation is not known. One of the molecular effectors of the AHR in COPD may be the NF-κB protein RELB. We have previously shown in lung fibroblasts, key structural cells involved in pulmonary inflammation, that the AHR promotes the retention of RELB, a feature necessary for the AHR to suppress COX-2. The anti-inflammatory abilities of the AHR may also involve alterations in the expression and function of the RNA-binding protein HUR and the miRNA miR-146a. Both of these (i.e. HuR and miR-146a) suppress COX-2 expression but their expression and involvement in COPD are also largely unexplored.

Therefore, the overall goal of this thesis is to define the contribution and mechanism of action of the AHR pathway in regulating COX-2 in the context of COPD.

The specific aims were:

Aim 1: Evaluate if the expression of AHR and RelB in human lung fibroblasts differ between Control, Smoking and COPD patients;

Aim 2. Determine if COPD lung fibroblasts exposed to cigarette smoke increase COX-2 is due to low AHR expression/activity and RElB degredation;

Aim 3. Assess whether cigarette smoke alters/disrupts physical interaction of AhR and RelB.

Chapter 2 - Materials and Methods

2.1 Subjects

Parenchymal lung tissue was obtained from individuals undergoing lung resection surgery for suspected lung cancer at McMaster University. Parenchymal tissue for the derivation of fibroblasts was taken from cancer-free sections of the lung. Recruited individuals included those with COPD, subjects without COPD but who were current or former smokers (Smokers) or non-smokers without COPD (Controls). Subjects were diagnosed based on the GOLD criteria for COPD. This study was approved by the Research Ethics Board of St Joseph's Healthcare Hamilton and all patients gave written, informed consent. The clinical features of the subjects used for the derivation of fibroblasts are given in Table 2.1. There was no significant difference between the three subject groups based on age. . There was also no significant difference in the length of smoking (pack years) between the Smoking and COPD groups.

2.2 Cell Culture

2.2.1 Derivation of primary lung fibroblasts

Human fibroblast strains were established from tissue explants placed under sterile coverslips in cell culture dishes containing RPMI (Invitrogen, Carlsbad, CA) supplemented with 20% FBS, 24.4mM Sodium Pyruvate, 2.4mM Non-Essential Amino Acids, 48.4mM L-Glutamine, 0.24M HEPES, 1.2mg/ml Gentamicin and 1.3mM β-Mercaptoethanol. Within 2 weeks of culture initiation, >95% of the cells were morphologically distinguishable as fibroblasts. Once the cells approached confluence,

they were detached with 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA) for 2 minutes at 37^0C. Trypsin was inactivated with an equal volume of 10% MEM and the cells were passed equally into 8- T_{75} flasks (BioLite™; Thermo Fisher Scientific, Waltham, MA). Fibroblasts within these flasks were allowed to grow to 90% confluence before being expanded into 12 x T_{175} flasks. Once 90% confluence was reached, the cells were collected using 0.05% trypsin, counted via hemocytometer and seeded into 6x T_{175} at a concentration of $2x10^6$ cells for continued expansion. The remaining cells were spun down at ~1000_xg and resuspended at a concentration of $2x10^6$ cells/ml in freezing media (50% MEM, 40% serum and 10% DMSO). One millilitre of cell suspension was aliquoted into Cryo-Tubes (Sarstedt, Nümbrecht, Germany) and frozen to -80^0C; remaining cells were subcultured in a T_{75} flask for experimentation and were used at the earliest possible passage. Unless otherwise indicated, all fibroblasts were plated at a density of 10,000 cells/cm^2.

2.2.2 U937 macrophages:

U937 macrophages were obtained from American Type Cell Culture (ATCC, Manassas, VA) and used for experiments designed to evaluate the interaction between AHR and RELB by co-immunoprecipitation. U937 cells were cultured in RPMI (Invitrogen, Carlsbad, CA) supplemented with 4.5g/L glucose, 25mM HEPES, 1.0mM sodium pyruvate, 10% FBS and 1% penicillin/streptomycin. U937 macrophages were cultured at 5% CO_2 at 37^0C, maintained at a density below $2.0x10^6$ cells/ml.

2.2.3 A549 cells:

A549 human lung epithelial cells were purchased from ATCC. A549 cells were used for characterization of fibroblasts and were cultured in 10% MEM media. Cell density of the A549 cells was kept below 2.0 x 10^6 cells/ml.

2.3 Fibroblast Characterization

Characterization of the primary lung fibroblasts was based on spindle shaped morphology, as well as the expression of markers determined by immunofluorescence (Section 2.4). Vimentin expression alongside the absence of cytokeratin, desmin, and α-smooth muscle actin (markers of epithelial cells, muscle cells and myofibroblasts, respectively) were used to determine cells as fibroblasts[52,54]. Antibodies were from Cell Signaling (Danvers, MA) and used at the following dilutions: vimentin:1:50; Desmin:1:25 and Keratin: 1:400 (in PBS with 3% bovine serum albumin (BSA)). Figure 2.1 displays the results of the immunofluorescent characterization and indicates purity of the fibroblast cultures.

2.4 Immunofluorescence

i) Immunocytochemistry - Human lung fibroblasts were seeded on 8-well glass chamber slides at a density of 8.0 x 10^3 cells/well and allowed to adhere for 24 h. Following serum starvation for 24 h, the cells were treated with media or 1% CSE, 10μM CH223191, or both 1% CSE and 10μM CH223191 for 4 or 24 hours to assess HUR localization or COX-2 expression. Following treatments, the cells were washed once with PBS, fixed

using 3% H_2O_2 for 15 min, and blocked with 5% normal horse serum for 2 hours at room temperature. Antibodies against HUR (Santa Cruz) and COX-2 (Cayman) were diluted 1:200 in PBS/BSA and incubated overnight at 4 °C. Levels of non-specific staining were assessed by incubating cells under identical conditions using the isotype- matched non-immune antibody (Santa Cruz) at the same concentration or by omission of the primary antibody. In all cases, the level of non-specific staining was negligible (data not shown). Anti-mouse or anti-rabbit IgG antibody Alexa Fluor® 555 (Invitrogen, Carlsbad, CA) was used for secondary binding (1:200) and incubated for 1 hour at room temperature. Slides were washed in PBS and incubated with Hoechst 33342 (Cell Signaling, MA; 1 ng/mL). Following a final wash, the slides were mounted using ProLong® Gold Anti Fade (Invitrogen, Carlsbad, CA), and viewed on an Olympus BX51 fluorescent microscope (Olympus, Ontario, Canada) and photographed using a Retiga 2000R Camera with ImagePro Plus software. All photographs were taken with identical exposure time and dynamic range.

ii) Immunofluorescence in Tissue - 1.5mm Thick paraffin tissue sections derived from lungs of Control, Smoker and COPD patients were washed in xylene three times for five minutes each. Slides were then washed in successive washes of 100%, 90% and 70% ethanol for 5 minutes. Sections were rinsed twice in PBS for 5 minutes each, permeablized with 0.2% Triton X100 in PBS for 30 minutes at room temperature and blocked using Dako Universal Blocking Solution (Dako, Carpinteria, CA) for 30 minutes at room temperature. Excess blocking solution was removed and the antibody against HUR protein added, (1:300 in Dako diluents, Dako, Carpinteria, CA) for 1 hour at room

temperature. Following three, 5-minute washes in PBS, Alexa Fluor 555 was diluted 1:1000 in Dako diluent and added for 1 hour at room temperature. Finally, Hoechst 33342 (Cell Signaling, MA; 1 ng/mL) was diluted 1:2000 in PBS and incubated on the slides for 15 minutes before being washed in PBS. ProLong® Gold Anti Fade (Invitrogen, Carlsbad, CA) was added directly to the tissue, followed by a glass cover slide. Slides were viewed on an Olympus BX51 fluorescent microscope (Olympus, Ontario, Canada) and photographed using a Retiga 2000R Camera with ImagePro Plus software.

2.5 Preparation of Cigarette Smoke Extract (CSE)

Research grade cigarettes (3R4F) with a filter were obtained from the Kentucky Tobacco Research Council (Lexington, KT). CSE was generated as previously described [43,160] by passing smoke from a single lit cigarette through 10ml of serum-free MEM (Life Technologies, Gaithersburg, MD) in a 50ml tube. Serum-free media was used in order to avoid serum induced expression of *Cox-2*. Each cigarette was burned to a distance of 1 cm above the filter over the course of 2 minutes. Stock CSE was quantified via spectrophotometric absorbance (Smart Spec-Plus™, Bio-Rad Laboratories, Mississauga, ON) at 320nm as compared against base media. A reading of 0.65AU was considered to represent 100% CSE. CSE was filter sterilized via a 0.2nm syringe filter, and the diluted to appropriate concentrations for treatment.

2.6 MTT Viability Assay

Fibroblasts from Control, Smoker, and COPD subjects were cultured in triplicate at a density of 8000 cells per well in 96 well flat bottom clear culture plates (Celltreat, Shirley, MA). Cells were allowed to grow to 90% confluence before treatment with CSE. Cells were treated with 140 µl of serial dilutions of CSE between 0.25- 8% CSE. Control wells were treated with only serum-free media. After 24 hours of treatment with CSE, 10ul of a 5mg/ml solution of MTT (Sigma-Aldrich, St. Louis, MO) and incubated at 37^0C for 4 hours. The plate was then centrifuged at 750RPM for 5 minutes. Following centrifugation, media was removed from the wells, and replaced with 200ul of DMSO. The plate was shaken gently by tapping the side in order to dissolve the MTT. The plate was spun at 750RPM for 10 seconds to remove DMSO from the sides of the wells and the absorbance was read at 510nm. Cellular viability was assessed relative to the untreated control for each[161–163] (Figure 2.2).

2.7 Analysis of Gene Expression

Total RNA was isolated from samples collected in 700µl of QIAzol trizol lysis reagent (Qiagen Inc., Valencia, CA). Using a Qiagen miRNeasy kit (Qiagen Inc., Valencia, CA), RNA was extracted as per manufacturer's instructions and quantified by spectroscopic analysis using a Nanodrop ND-1000™ (Thermo Fisher Scientific, Waltham, MA). RNA quality was considered sufficient by a comparison of the 260/280abs ratio, which ranged between 1.8 to 2.0. Samples were then diluted to an RNA concentration of 10ng/ml in nuclease-free water. Reverse transcription was carried out in a 10-µl reaction mixture by

iScript II™ Reverse Transcription Supermix (Bio-Rad Laboratories) at 25°C for 5 min followed by 42 °C for 30 min. Real time PCR (qPCR) was performed with 1 µl cDNA and 0.5 µM primers in Sosofast™ Eva Green® Supermix (Bio-Rad Laboratories) and PCR amplification was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Primer sequences used for human *Cox-2* were TCA CAG GCT TCC ATT GAC CAG (fw) and CCG AGG CTT TTC TAC CAGA (rv). *RelB* primer sequences were TGT GGT GAG GAT CTG CTT CCAG (fw) and GGC CCG CTT TCC TTG TTA ATTC (rv). *Cyp1b1* primer sequences were TAT AGA CAC ATA CAC CCA AAC ACT TAC ACC (fw), CCT TAT CAC CTT CAA TCA CAC ACT TTA CAC (rv). Primer sequences for β-actin were CTA CAA TGA GCT GCG TGT G (fw) and TGG GGT GTT GAA GGT CTC (rv), and for GAPDH were GTC TCC TCT GAC TTC AAC AGC(fw), ACC ACC CTG TTG CTG TAG CCA(rv). Melt curve analysis was performed to ensure that nonspecific products were absent. The fluorescence detection threshold was set above the non-template control background within the linear phases of PCR amplifications and the cycle threshold (Ct) of each reaction was detected. Results are presented as fold-change change in Ct normalized to housekeeping β-actin or GAPDH. Expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

2.8 Analysis of microRNA expression

Total RNA, including miRNA was extracted as described above and miRNA expression assessed by two-step TaqMan® RT-PCR (Applied Biosystems, Carlsbad, CA) for miR-146a and U6 snRNA, a small nuclear RNA (snRNA) used as an internal control. miRNA expression was normalized to the U6 snRNA levels and fold-change was determined

using $2^{-\Delta\Delta Ct}$ method.

2.9 RelB-siRNA knock-down in lung fibroblasts

Control fibroblasts were seeded at 1-2 x 10^4 cells/cm^2 and transfected with 40-60 nM of siRNA against *RelB* (Santa Cruz, sc-36402 human) or non-targeting control siRNA (Santa Cruz, sc-37007) according to manufacturer's instructions with the following exceptions. After six hours past the initial transfection, 1ml of serum free media was added to the cells and incubated at 37^0C for an additional 18 hours. Cells were then treated with 2% CSE or IL-1β for 6 hours and RNA or protein was collected for further analysis. Verification of *RelB* knock-down was done by western blot within 72 hours after transfection.

2.10 Western Blot

Total cellular protein was prepared by washing cells with sterile PBS and lysing with 1% IGEPAL buffer supplemented with 10% protease inhibitor cocktail (leupeptin, aprotinin, pep- statin, and PMSF; Sigma-Aldrich, St. Louis, MO). Cells were scraped with a Corning scraper (Corning Inc., Corning, NY) and transferred to a 1.5ml tube for 30 minutes on ice and occasionally vortexed to facilitate for lysis. Tubes were centrifuged at >17,000_xg for 10 minutes (4^0C). Following centrifugation, the supernatant was collected and transferred to a new 1.5ml tube. Protein amounts were quantified by BCA assay (Thermo Scientific. Rockfort, IL) and 5-10 µg of protein were resolved on SDS-PAGE gels and electro-blotted onto Immun-blot PVDF membrane using transfer buffer (48 mM

Tris, 39 mM glycine, 20% methanol in a 1L volume, pH 9.2) and a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories). Antibodies against AhR (1:1000; Santa Cruz Biotechnology), RelB (1:1000; Santa Cruz Biotechnology) and Cox-2 (1:1000; Cayman, Ann Arbor, MI) were used to assess changes in expression and normalized against β-actin (1:10,000; Millipore, Billerica, MI). Proteins were visualized using HRP-conjugated secondary antibodies (1:8000) followed by Clarity™ enhanced chemiluminescence (ECL) substrate (Bio-Rad Laboratories) and detected with an electronic imager (Alpha Innotech, San Leandro, CA).

2.11 Co-Immunoprecipitation

Human lung fibroblasts from Control subjects at 75% confluence in 1 T-175 flask per treatment were scraped with a Corning cell scraper (Corning Inc.) in 10mL ice cold PBS and transferred to a 15mL tube and then centrifuged at 3000 RPM for 15 minutes at 4^0C. Supernatant was removed, leaving a cell pellet. U937 macrophages were collected from culture flasks and transferred to 15mL tubes before being centrifuged at 1000 RPM for 6 minutes, whereupon the supernatant was removed. The U937 cells were resuspended in ice cold PBS before being centrifuged again at 3000 RPM (4^0C) for 15 minutes to again form a cell pellet. Cell pellets from either U937 or Control subjects were re-suspended in 1mL of CoIP lysis buffer (1% IGEPAL lysis buffer, supplemented with 10µM $Na3VO_4$, 10µM PMSF, 100µM DTT), vortexed and incubated while rocking on ice for 30 minutes. Lysates were centrifuge at 13000 RPM for 10 minutes at 4^0C and the supernatant collected. 100µl of the lysate was kept for protein quantification. The remaining lystate was incubated with 10µl of AhR antibody (Santa Cruz Biotechnology) or 2µl of IgG

isotype control antibody (Santa Cruz Biotechnology) together with 30uL of Protein G beads (Pierce, Rockfort, IL) overnight at 4^0C with constant agitation. Tubes were then centrifuged at 6000 RPM for 10 minutes at 4^0C. The supernatant was removed and the pellet was resuspended with 1mL 1% IGEPAL lysis buffer (without supplement). Tubes were centrifuged for 5 minutes at 6000 RPM, and washed twice with PBS at 6000 RPM for 5 minutes each. The supernatant was removed and the beads were resuspended in 30uL of sample buffer. Each tube was boiled at 95^0C for 5 minutes and the sample was loaded for SDS-PAGE when cooled.

2.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc.). An analysis of variance (ANOVA) test was used to assess significance between treatment group means of more than two. Newman-Keuls post-hoc test was used to compare means of specific groups. Results are expressed as the mean ± SEM. In all cases, a p-value < 0.05 was considered statistically significant.

Table 2.1. Fibroblast explant patient characteristics

	Control	Smoker	COPD
n of subjects	6	15	12
Age (yr)	66.7±2.6	64.0±0.7	68.3±0.9
Gender (M/F)	(2/4)	(8/7)	(8/4)
Time Smoking*	0.0	36.5±1.2	37.6±1.3
FEV1 (%)	88.2±3.3	88.2±0.9	**65.8±2.2**
FVC (%)	83.0±3.4	92.0±0.9	81.4±2.3
FEV1/FVC (%)	88.0±2.7	76.6±0.5	55.0±1.7
Results are presented as mean ± SEM			
*** denotes pack-years**			

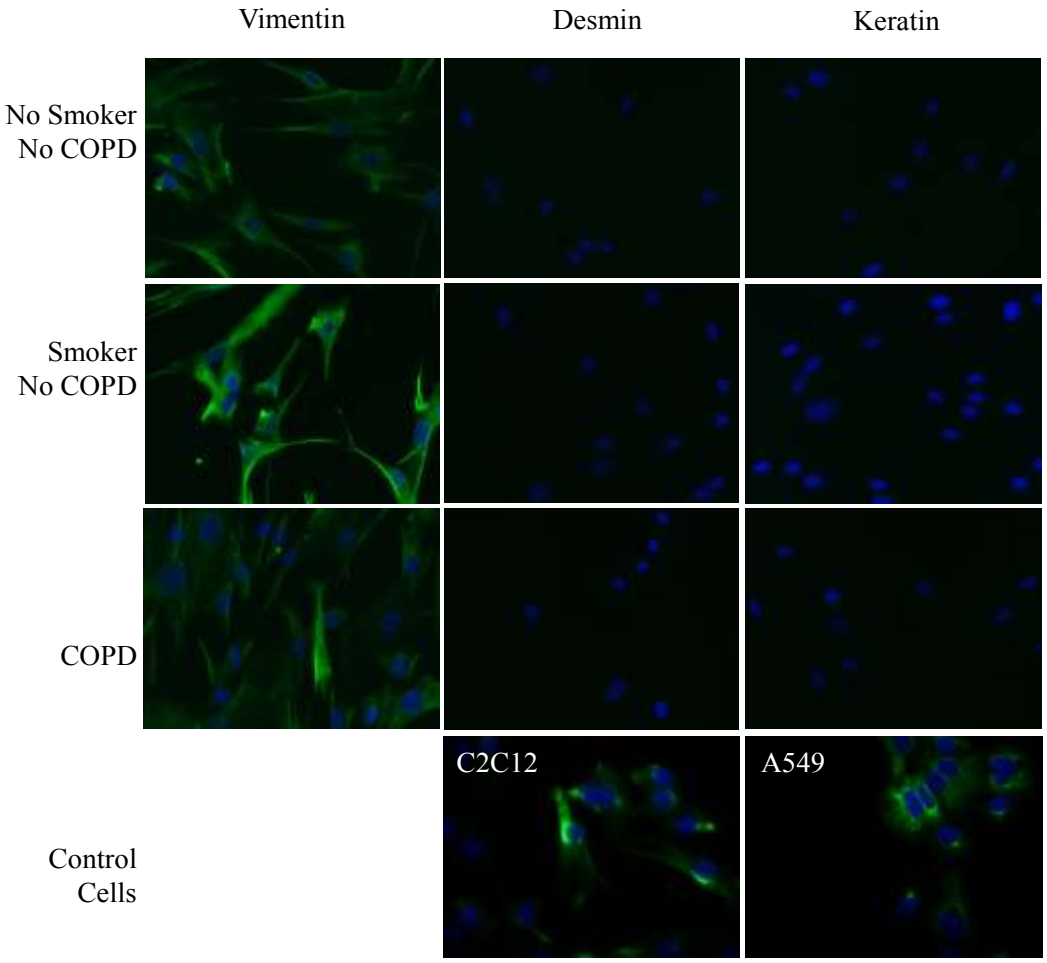

Figure 2.1 Characterization of explant fibroblasts **-** Immunofluorescent staining of human lung fibroblasts derived from No smoker/ no COPD (control), Smoker/No COPD, and COPD subjects. Target proteins were visualized with fluorescein (green) in combination with Hoechst (blue) nuclear stain. C2C12 myoblasts and A549 epithelial cells were used as positive controls for desmin and keratin respectively. Each subject type was stained with antibodies specific to vimentin (left), desmin (middle), and keratin (right). All human lung fibroblasts were positive for vimentin, but negative for desmin and keratin.

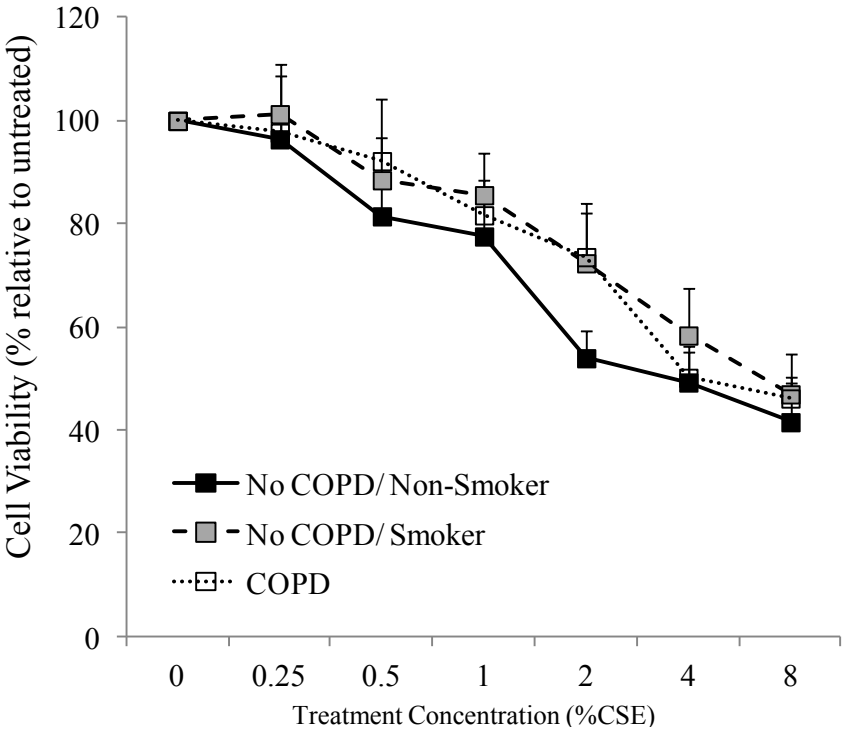

Figure 2.2. MTT viability of human lung fibroblasts. Cell viability after treatment with CSE. Cellular viability decreases in a dose dependant manner. No significant differences are found between patient groups except at 2% CSE, where the No COPD/ No Smoke is significantly less viable than other groups. All strains exhibit >80% viability when treated with 1% CSE, the treatment condition used other experiments (N=6).

Chapter 3 - The Aryl Hydrocarbon Receptor regulates COX-2 expression via HUR

3.1 Results

3.1.1 AHR expression is reduced in COPD-derived fibroblasts:

The AHR is expressed in most tissues, and is particularly well-expressed in the lung[128]. The AHR profoundly regulates inflammation in the lung in response to cigarette smoke, including the expression of COX-2[142,160]. Reduced AHR expression occurs in inflammatory conditions such as inflammatory bowel disease (IBD)[138], but the expression of AHR in cigarette smoke-induced lung diseases, including COPD is not known. To begin to explore the connection between the AHR and COPD, we measured the baseline expression of AHR protein in fibroblasts derived from Control (never smokers), Smokers (current and ex-smokers) and COPD subjects by western blot. Figure 3.1 shows a significant reduction of AHR protein in COPD patient-derived primary lung fibroblasts.

3.1.2 COX-2 protein, but not mRNA, is elevated in COPD derived lung fibroblasts:

Patients with COPD exhibit increased production of inflammatory mediators, including increased expression of pulmonary COX-2[39,49]. As lung fibroblasts are a robust source of COX-2[32], we next assessed for differences in the expression of *Cox-2* mRNA and protein between Control, Smoker and COPD-derived fibroblasts. Consistent with previous reports[164], COPD-derived lung fibroblasts expressed more COX-2 protein than either Control or Smoking subject fibroblasts (Figure 3.2A). There was no significant

difference in the expression of *Cox-2* mRNA between Control, Smoker and COPD-derived lung fibroblasts (Figure 3.2B).

3.1.3 Inhibition of the AHR potentiates the effect of CSE on COX-2 expression:

To now explore the connection between the AHR and COX-2 production, the expression of *Cox-2* mRNA was measured after treatment with CSE, with and without the presence of the AHR antagonist CH-223191. Figure 3.3A shows that 1% CSE did not upregulate *Cox-2* mRNA after 24 hours. Inhibition of the AHR without 1% CSE resulted in an increase in *Cox-2* mRNA in both the Control and Smoker-derived lung fibroblasts, but not lung fibroblasts derived from COPD subjects. Inhibition of the AHR with CH-223191, together with 1% CSE further increased the expression of *Cox*-2 mRNA in Control and Smoker-derived fibroblasts. CH-223191 with 1% CSE did not significantly increase *Cox-2* mRNA produced by COPD derived fibroblasts. This is consistent with the lack of AHR seen in the COPD-derived fibroblasts (Figure 3.1).

Next, we evaluated *Cyp1b1* mRNA, a marker of classic activation of the AHR[165,166]. One hour pre-treatment with CH-223191, with or without 1% CSE significantly reduced the expression of *Cyp1b1* mRNA in only Control subject derived fibroblasts (Figure 3.3B). There was no significant difference between CH-223191 treated fibroblasts, or fibroblasts treated with 1% CSE and CH-223191

3.1.4 COX-2 expression in lung fibroblasts coincides with HUR expression and localization:

The absence of *Cox-2* mRNA induction and the presence of COX-2 protein (Figure 3.2) suggested COX-2 was post-transcriptionally regulated. To better determine the role of the AHR in regulating COX-2, we measured if HUR expression is affected by treatment with CSE with and without CH-223191. HUR is a member of the ELAVL1 family of proteins that translocates from the nucleus to the cytoplasm upon activation where it stabilizes target mRNA transcripts that possess a 3'-UTR sequence containing an AU rich element (ARE)[113] such as COX-2[88]. Figure 3.5 demonstrates that the expression of HUR was not significantly different in lung fibroblasts derived from three different Controls. Treatment with 1% CSE with or without CH-223191 did not change HUR expression at either 6 or 24 hours.

We next investigated whether inhibition of AHR activity influenced HUR translocation to the cytoplasm, a key factor in the ability of HUR to stabilize target mRNA[88]. Figure 3.6 details the effect of CSE and CH-223191 on Control lung fibroblasts after 4 hours of exposure. Treatment with 1% CSE induced cytoplasmic translocation of HUR compared to the media-only (Figure 3.6, *bottom left)*. Inhibition of AHR by CH-223191 also induced detectable cytoplasmic HUR (Figure 3.6, *top right*). However, co-treatment of CSE with CH-223191 dramatically affected HUR translocation (Figure 3.6, *bottom right*). This suggests that the AHR is involved in retaining HUR within the nucleus, and may account for the increased COX-2 protein expression in response to cigarette smoke.

3.1.5 HUR cytoplasmic localization is increased in Smoker and COPD subject lung tissue:

Finally, we investigated HUR expression and localization in lung tissue, as to date, there is little information about HUR in COPD subjects. Figure 3.7 shows three representative patients for Control, Smoker and COPD subjects. Cytoplasmic HUR was only observed in Smoker and COPD subjects (Figure 3.7). Arrows indicate nuclei that do not exhibit translocation, and are seen in both the Control and Smoker subjects. This data indicates that in Smoker and COPD subjects the amount of HUR retained in the nucleus is decreased. Smoker lung tissue presents a more intermediate phenotype, as less HUR is cytoplasmically-localized.

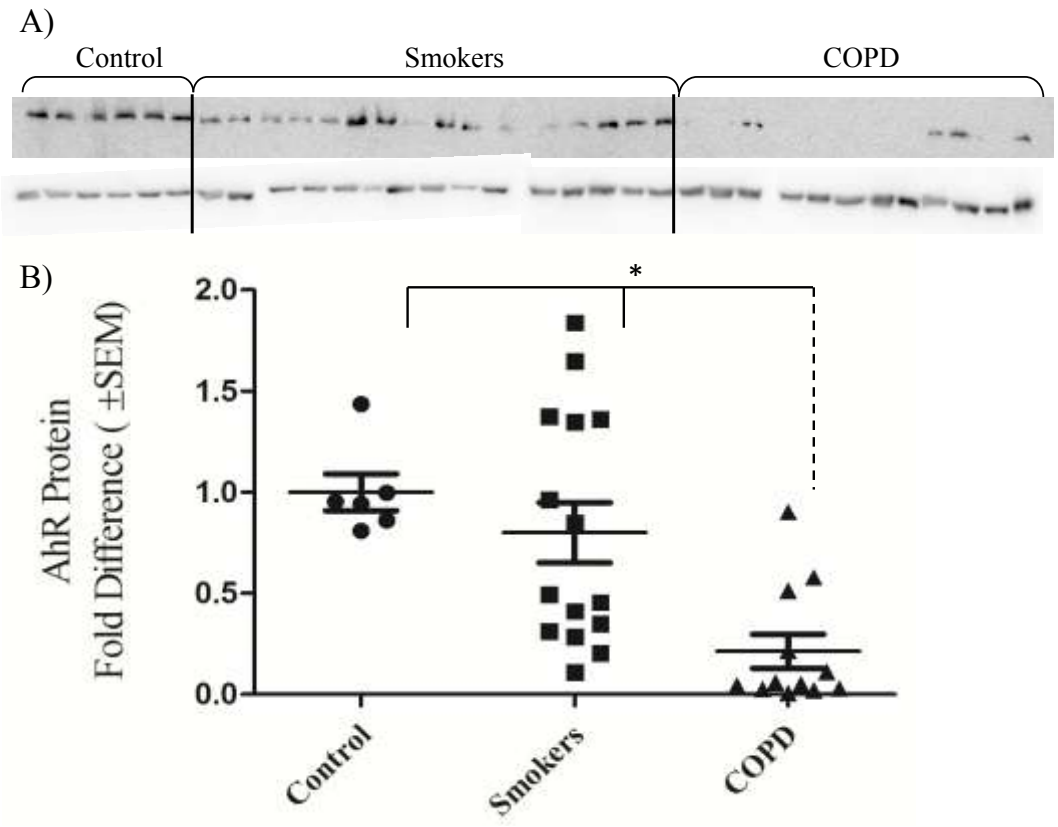

Figure 3.1 Expression of AHR protein is decreased in COPD derived fibroblasts. A) Fibroblasts derived from Non-Smokers (Control, N=6), Smokers (N=15), and COPD (N=12) subjects were cultured to 90% confluence, and serum starved for 24 hours before harvesting cell lysates for western blot. AHR protein expression was significantly reduced in the COPD derived subject fibroblasts as compared to the control or smoking subject fibroblasts. B) Densitometry for AHR western blot in A. Each point represents an individual subject.

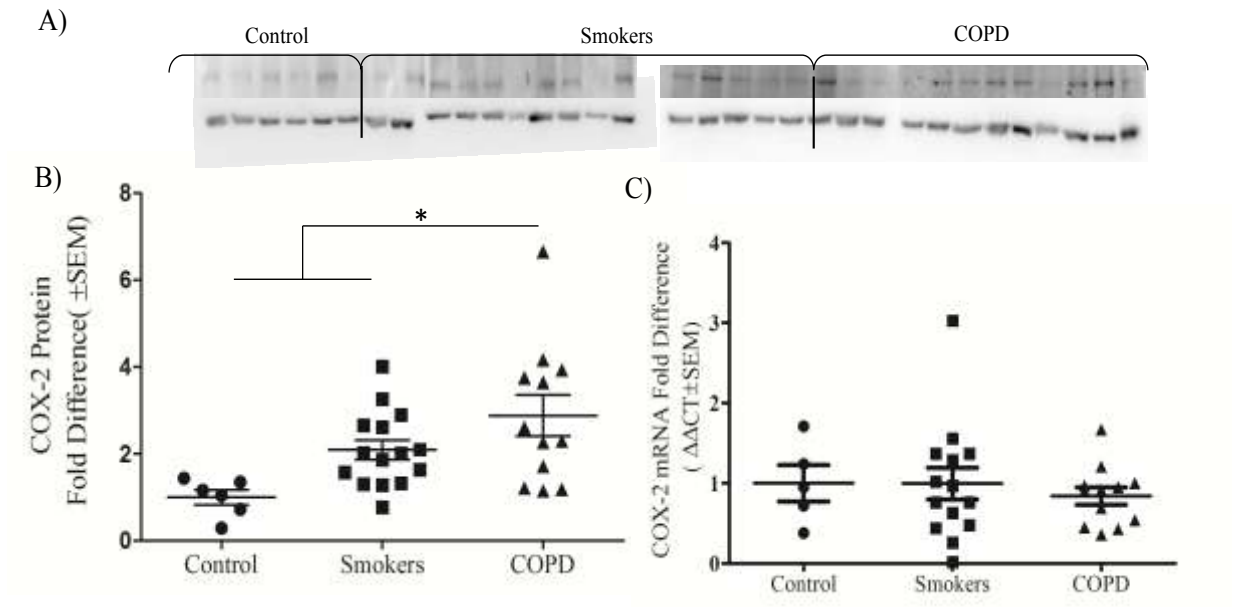

Figure 3.2. COPD lung fibroblasts have higher baseline COX-2 protein but not mRNA. A) Increased COX-2 protein was observed in lung fibroblasts derived from Smokers and COPD subjects. B) Densitometry- There is a significant increase in basal COX-2 protein expression in lung fibroblasts from COPD subjects (** $p < 0.001$) and Smokers (*** $p < 0.01$). C) There was no difference in basal COX-2 mRNA levels between Control, Smokers and COPD lung fibroblasts.

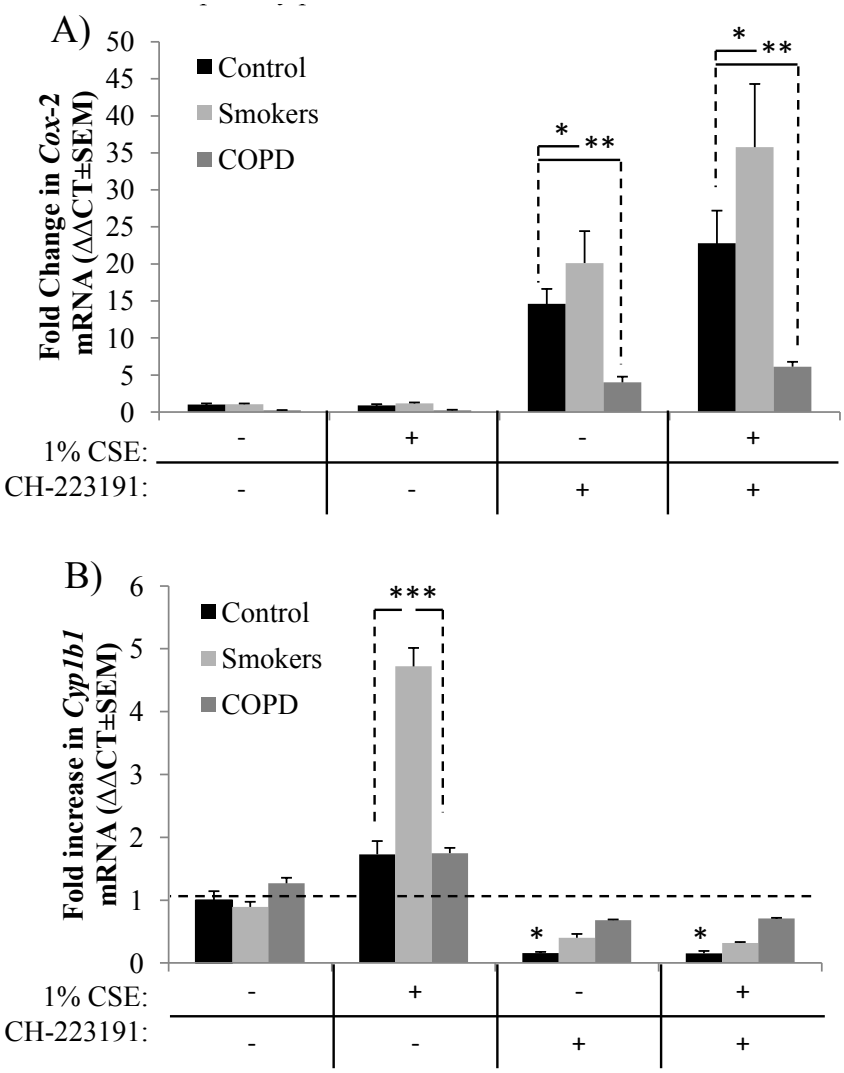

Figure 3.3. A) Lung fibroblasts treated with 1% CSE for 24 hours did not significantly increase *Cox-2* mRNA expression in all patient groups. Treatment with AHR antagonist CH-223191 (10μM) for 24 hours significantly increased *Cox-2* mRNA in Control and Smokers, but not in COPD subject derived fibroblasts ($p \leq 0.01$). Pre-treatment with antagonist for 1 hour, followed by co-treatment with 1% CSE for 24 hours further increased the *Cox-2* expression in Control and Smoker groups, but did not affect COPD derived fibroblasts. B) Only in Smoker derived fibroblasts was significant ($p \leq 0.001$) activation of the *AHR* seen in response to cigarette smoke. All subject derived fibroblasts showed a significant reduction in *Cyp1b1* mRNA expression when inhibited by the *AHR* antagonist CH-223191 (10μM). Although still significant, the inhibition effect of CH-223191 was reduced in smokers, and further reduced in COPD derived fibroblasts. (n=3 for each group)

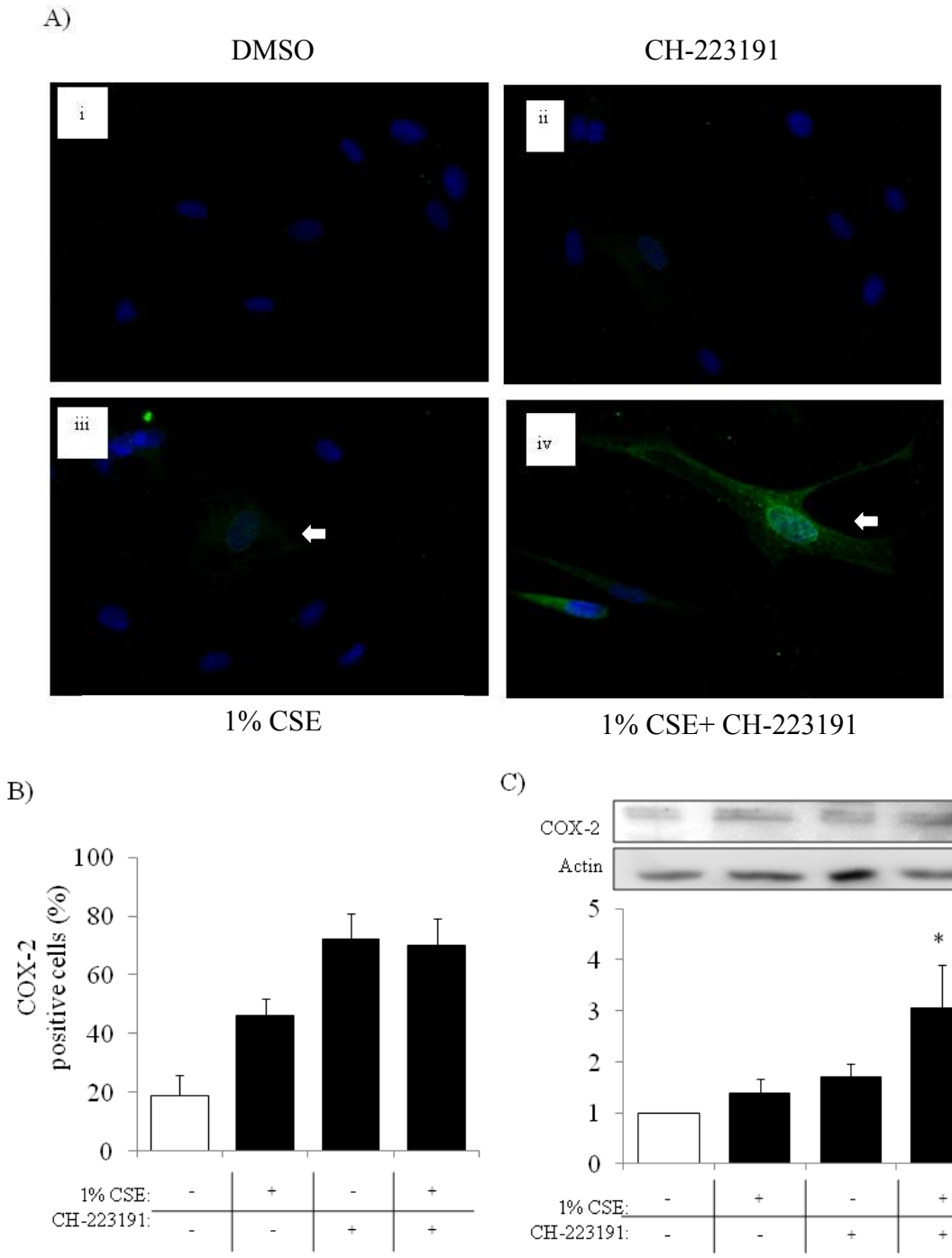

Figure 3.4. COX-2 expression in human lung fibroblasts is elevated by inhibition of the AHR. A) There was a small increase in COX-2 protein in CSE-exposed human lung fibroblasts. Cells exposed to both 1% CSE and CH-223191 (10μM), show increased induction of COX-2 (arrows). B) There was a significant increase in the percentage of COX-2-positive human lung fibroblasts in response to CH-223191 and exposed to CSE (** $p < 0.01$). C) There was a significant induction in COX-2 protein in human lung fibroblasts exposed to 1% CSE in conjunction with CH-223191 (3.1 ± 0.84; * < 0.05 compared to both media control and 1% CSE alone). A representative western blot is shown of experiments utilizing fibroblasts from three different (N=3) individuals.

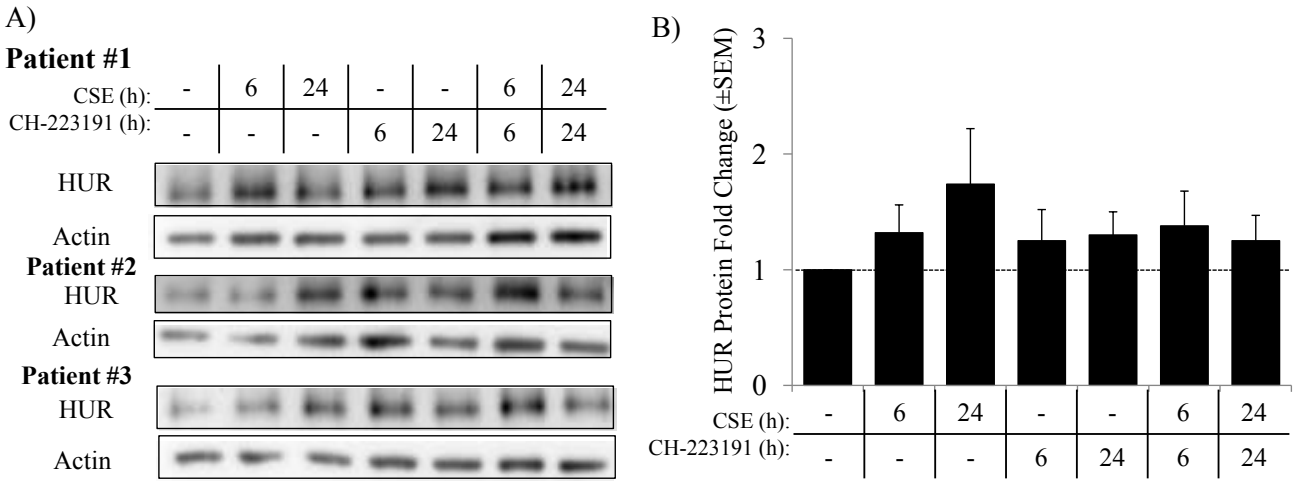

Figure 3.5. HUR protein expression is not significantly altered by treatment with 1% CSE or AHR agonist CH-223191. A) Primary human lung fibroblasts from three non-smoking individuals express HUR and the relative expression level was not altered by exposure to CSE or inhibition of AHR activity. B) Densitometric analysis indicated that there was a slight but not statistically significant change in HUR protein expression in response to CSE for 24 hours. Exposure to the AHR antagonist CH-223191, with or without CSE, also did not significantly alter HUR protein expression.

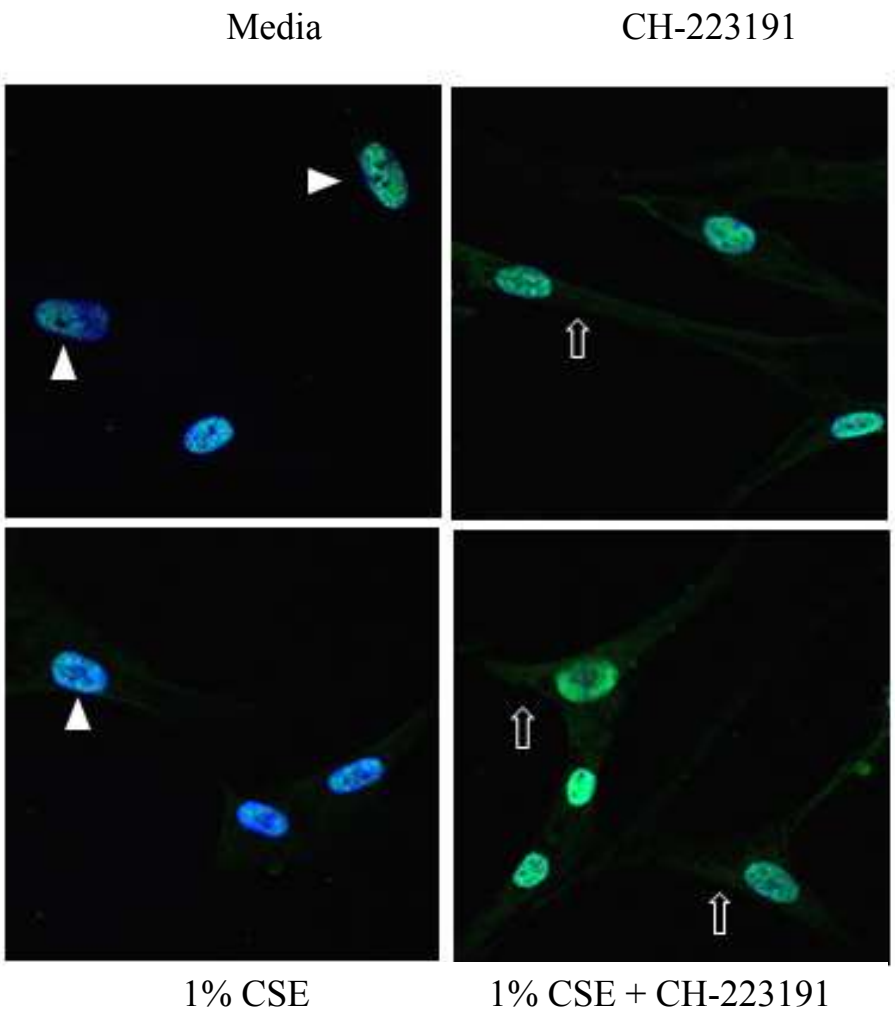

Figure 3.6. HUR cytoplasmic shuttling increases with inhibition of the AHR. HUR localization (green) at 4 hours post treatment was assessed by immunofluorescent staining using Alexa-555 fluorescently tagged antibody. primarily restricted to the nucleus (blue) in human lung fibroblasts exposed to media, and only a slight increase in translocation to the cytoplasm was seen with 1% CSE treatment(*top and bottom left, arrowheads*). Inhibition of AHR activity with CH-223191(10μM) slightly increased the amount of HUR in the cytoplasm *(top right, open arrows).* Pretreatment with CH-223191 followed by 1% CSE increased HUR cytoplasmic shuttling (bottom right, *open arrows*).

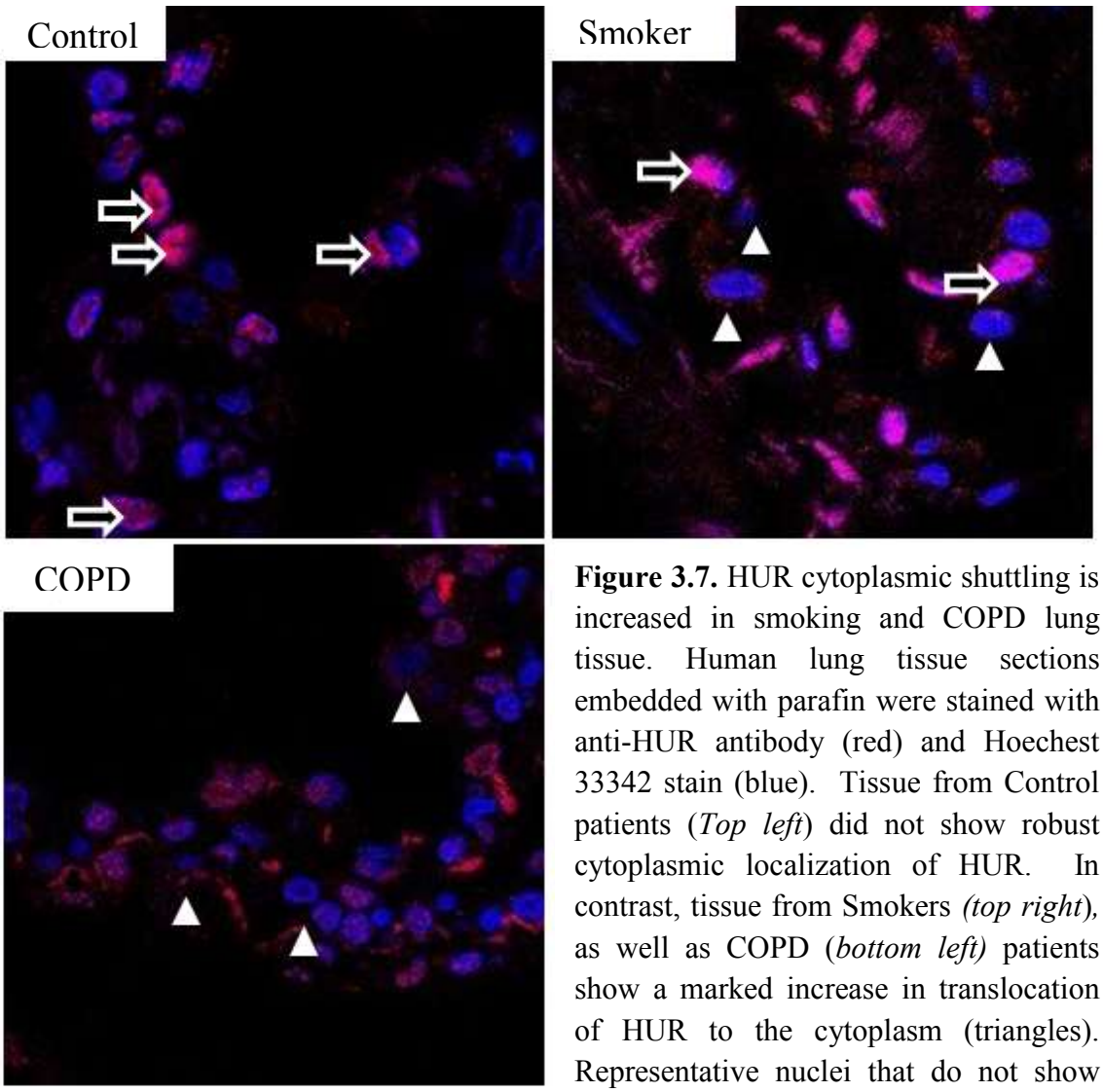

Figure 3.7. HUR cytoplasmic shuttling is increased in smoking and COPD lung tissue. Human lung tissue sections embedded with parafin were stained with anti-HUR antibody (red) and Hoechest 33342 stain (blue). Tissue from Control patients (*Top left*) did not show robust cytoplasmic localization of HUR. In contrast, tissue from Smokers *(top right*), as well as COPD (*bottom left)* patients show a marked increase in translocation of HUR to the cytoplasm (triangles). Representative nuclei that do not show translocation are indicated as well (arrows). (n=3 for each group)

3.2 Discussion

One of the goals of this study was to extend the research on the AHR as a regulator of inflammation, particularly as it relates to the human lung and cigarette smoke-induced pathology. Therefore, we analyzed AHR expression in Control, Smoker and COPD subject-derived fibroblasts. We report that the expression of the AHR is decreased in COPD patient-derived fibroblasts, and the activity of the AHR is critical to the suppression of both COX-2 mRNA and protein. Furthermore, suppression of COX-2 is linked to AHR regulation of HUR protein, an RNA binding protein able to stabilize *COX*-2 mRNA. To date, little has been reported about the AHR in the adult human lung and therefore the significant reduction in AHR expression in COPD patient-derived fibroblasts represents a novel finding (Figure 3.1).

The mechanism by which AHR is reduced in COPD is not known. Curiously, there is some variation in AHR expression in lung fibroblasts derived from Smokers, suggesting that reduced AHR expression may indicate the risk of eventually developing COPD. Longitudinal studies of smoking subjects could offer more insight to the progression of emphysema and COPD with relation to AHR expression over time. Thus, in those individuals who smoke and have low AHR, may be at an increased risk for eventually developing COPD. It is known that subsequent to activation, the AHR is degraded in a proteasome-dependant manner[167,168] and therefore the reduction in AHR seen in COPD subject derived lung fibroblasts may be a result of chronic activation by cigarette smoke. Other mechanisms that may account for reduced expression of the AHR may be due to epigenetic alterations[169–171]. Chronic smoke induced chromatin remodelling[172] or epigenetic silencing via histone modification[125,169,170,173–175] are often

mediated by increased DNA methyltransferase expression with subsequent gene methylation[176,177]. Methylation of the AHR promoter, and the repression of DRE-mediated genes via the Dnmt3B DNA methyltransferase has been reported[178]. The presence of known polymorphisms in the AHR gene[128,179] may also contribute an innate susceptibility to the loss of AHR. Previously, AHR polymorphisms have been associated with varied disease susceptibility, ranging from male infertility to lung cancer[180–183]. These polymorphisms were predominately found outside of the ligand binding region[179,180] but have been reported to affect ligand binding affinity[184,185] and may have effects on the degradation rate or stability of the AHR.

The connection between COPD and elevated COX-2 expression has been well established[33,93,186], and previous studies of AHR-deficient mice show increased expression COX-2 protein in response to smoke [106,142]. Additionally, pharmaceutical inhibition of COX-2 via celecoxib was shown to halt the progression of emphysematous destruction of lung tissue in rats[100], suggesting *Cox-2* expression may be an important contributor to the progression of COPD. COX-2 protein expression is elevated in COPD derived lung fibroblasts (Figure 3.2A,B) but the lack of a difference in *Cox-2* mRNA expression between Control, Smoker and COPD subject derived fibroblasts was notable (Figure 3.2C). These results suggested that COX-2 protein expression may not rely solely on transcriptional regulation to control the levels of this inflammatory mediator. Indeed, several post-transcriptional mechanisms are involved in COX-2 regulation.

Interestingly no difference in *Cox-2* mRNA expression was observed when patient derived fibroblasts were treated with CSE for 24 hours (Figure 3.3A). Subsequent analysis at 3 and 6 hours revealed this difference however (the reader is referred to

Chapter 4) (Figure 4.3A). In both the Control and Smoker derived lung fibroblasts, competitive inhibition of AHR activation by CH-223191 significantly increased *Cox-2* mRNA production. Concurrent treatment with CSE and CH-223191 induced *Cox-2* mRNA, an effect was blunted in the COPD lung fibroblasts. These data suggest that AHR activity is involved in the regulation of *Cox-2* mRNA in human lung fibroblasts. It may be that the blunted effect seen in the COPD-derived lung fibroblasts is due to the significant reduction in AHR protein expression in the COPD lung fibroblasts. This may have prevented CH-223191 from having as prominent an effect, as there was limited AHR remaining. This observation was reminiscent of baseline *Cox-2* mRNA, which was not increased in the COPD derived lung fibroblasts. Further studies using additional commercially available inhibitors of the AHR, such as 6,2',4'-Trimethoxyflavone or α-NF could confirm these findings[187,188]. These additional inhibitors may also illuminate the class of AhR activity required, as CH-223191 antagonism has been reported to be preferential for halogenated aromatic hydrocarbons (HAHs)[188].

CH-223191 inhibited *Cyp1b1* mRNA induction, an effect that was dependent on cells which expressed abundant AHR protein. Here, the ability of CH-223191 to attenuate *Cyp1b1* mRNA was reduced in the COPD derived fibroblasts (Figure 3.3B), consistent with lower AHR expression. It is interesting that CSE significantly increased Cyp1b1 in the Smoker fibroblasts compared to Control. and may suggest prolonged AHR activation or stabilization of *Cyp1b1* mRNA. Stabilization of *Cyp1b1* mRNA may occur in Smokers due to metabolism of accumulated toxic components of smoke like B[*a*]P[37,89,165]. One proposed link between *Cyp1b1* mRNA stability is the expression of miR-27b[189]. This mechanism is of potential interest in light of recent research on miRNAs in disease[93,190],.

Cyp1b1 mRNA induction by CSE was absent in Control and COPD subject derived fibroblasts at 24 hours (Figure 3.3B), suggesting prolonged activation of *Cyp1b1* is an effect of long term smoke exposure. Collectively, these results suggest that AHR activity is an important aspect of the suppression of *Cox-2* mRNA.

To understand if the AHR regulates COX-2 protein in addition to mRNA, Control patient derived lung fibroblasts were treated with CSE and CH-223191 (Figure 3.4). Combination treatment of CSE and CH-223191 for 24 hours substantially increased both the amount of COX-2 positive cells (Figure 3.4B) and the relative amount of COX-2 protein (Figure 3.4C). These data are consistent with the effect of combination CSE and CH-223191 treatment on *Cox-2* mRNA production (Figure 3.3A). One difference between the production of COX-2 protein and that of mRNA is the effect of AHR inhibition by CH-223191. Although treatment with CH-223191 was able to upregulate the expression of *Cox-2* mRNA , it did not significantly increase the amount of COX-2 protein (Figure 3.4). The presence of *Cox-2* mRNA and the absence of protein expression suggested translation of COX-2 protein was prevented by another as yet unknown factor. CSE in combination with CH-223191 did increase COX-2 protein expression, suggesting this factor was influenced by the presence of CSE. Taken together, these data suggested a link between CSE and post-transcriptional regulation of COX-2 protein.

Post transcriptional regulation can occur by controlling nuclear import/export, translation initiation and mRNA stability[191,192]. This can be accomplished by multiple methods, including but not limited to, RNA binding proteins (RBPs)[111,193], miRNA expression[114,190] or the recruitment of translation initiation factors[194]. We chose to

investigate whether RBPs were the mechanism through which the AHR may regulate COX-2 based on ample evidence for RBP-mediated regulation of COX-2 protein expression[88,101,103,195]. In particular, the RBP HUR represented a strong link between the AHR and *Cox-2* mRNA, as HUR is known to stabilize *Cox-2* mRNA[101]. Regulation of RBP localization in response to stimuli is an important mechanism, as many RBPs shuttle between the nucleus and the cytoplasm in response to varied cellular stresses [103,196]. We therefore hypothesized that the AHR plays an important role in determining either the expression or localization of HUR in response to CSE. We report that HUR protein expression is not changed significantly after 1% CSE treatment, with or without CH-223191 inhibition of the AHR (Figure 3.5). This data excluded increased HUR expression as a mechanism by which COX-2 expression was regulated via the AHR.

Although the expression of HUR did not significantly change after CSE or CH-223191 exposure, HUR localization was significantly affected. CSE treatment resulted in detectable HUR translocation to the cytoplasm, and inhibition of the AHR resulted in a visible increase in cytoplasmic HUR (Figure 3.6A). Combination treatment of CSE and CH-223191 were observed to act synergistically, increasing HUR protein localization to the cytoplasm significantly. How the AHR regulates the localization of HUR is unknown, but there are several possible explanations. First, the interaction between AHR and HUR may be direct and inhibition of the AHR (thereby preventing AHR from reaching the nucleus), would prevent a potential AHR/HUR complex that sequesters HUR directly. Such an interaction may involve direct AHR/HUR binding, or be mediated in complex with known accessory proteins such as the ARNT, HSP90 or XAP2[197]. AHR activity may also prevent HUR translocation via control of upstream signalling cascades, such as the

p38-MAPK. Recent studies have shown inhibition of p38 (or its target MAPKAPK-2) increased cytoplasmic HUR and increased *Cox-2* mRNA stability in human mammary cells[198]. Furthermore, AHR activates p38-dependant genes such as c-jun [199,200] and inhibition of p38 (via compounds SB203580 and SB202190[201]) suppresses the expression of TCDD-induced *Cyp1a1* and *Cyp1b1*[201], indicating reciprocal regulation between p38 and AHR. When considered together with our data, this suggests the possibility that inhibition of the AHR increases HUR cytoplasmic shuttling by inhibition of p38. The mechanism by which p38 regulates HuR may be p38 phosphorylation of HUR[202]. Additionally, phosphorylation of TTP by p38 decreases TTP affinity for the ARE and prevents it from competing with HUR at the ARE[203]. Both mechanisms result in HUR-mediated stabilization of mRNA. Finally, the AHR may affect HUR shuttling through control of HUR export from the nucleus. It has been recently suggested that HUR export from the nucleus operates in concert with mRNA export, and that HUR binds transcripts in order to facilitate mRNA movement to the cytoplasm[101,204,205]. Chromosome maintenance region 1 (CRM1), and transportin-2 have been suggested to contribute HUR shuttling[205,206]. Transportin 2 has been shown to control nuclear import of HUR[207,208]. Additionally, caspase mediated clevage of HUR during apoptotic conditions interfered with transportin 2 import of HUR, allowing for cytoplasmic accumulation[209]. No information concerning the AHR and transportins has been reported, so this represents a novel area for future investigation.

Finally, immunofluorescent staining of HUR in human lung tissue from Control, Smoking or COPD patients (Figure 3.7) expands the link between the AHR and HUR to the pathology of COPD. Sub-cellular localization of HUR from nuclear (Control), to an

intermediate phenotype (Smokers) and finally to predominantly cytoplasmic (COPD), provides some information that the reduction in AHR expression observed in COPD has an observable effect on the translocation of HUR in actual patient tissue. HUR protein localized in the cytoplasm of COPD lung tissue may explain the increase in the baseline levels of COX-2 protein, as increased cytoplasmic HUR will increase the amount of COX-2 protein produced independent of additional *Cox-2* mRNA (Figure 3.2).

In conclusion, this research has helped elucidate the physiological role of the AHR, supporting the notion that the AHR is not only able to initiate toxic response genes, but also controls inflammation. Collectively, our data show that AHR expression and activity are required for the suppression of COX-2 produced in response to cigarette smoke exposure, and suggest increased HUR cytoplasmic shuttling as a result of AHR inhibition as the mechanism through which this is accomplished. We also demonstrate the potential clinical relevance of this mechanism in COPD. Future studies to confirm that HUR is increasing the stability of the COX-2 mRNA in response to AHR inhibition and CSE treatment should be done by inhibiting general transcription via actinomycinD and analyzing *Cox-2* mRNA remaining after time. As previously suggested, the p38-MAPK pathway represents a good target for future investigations based on how it has shown to be regulated by AHR, and to regulate COX-2 mRNA in a HUR mediated manner[198,199]. Inhibition of p38 in fibroblasts with concomitant treatment of CSE and CH-223191 will reveal if the AHR is regulating HUR shuttling via this pathway. With this research we hope to highlight the AHR as a critical point of study in the future of inflammatory research, and explore the AHR's connection to other proteins regulating inflammation. One such protein, RELB, is degraded in the presence of smoke when AHR

expression is absent[142,210]. As such, exploring how RELB contributes to the regulation of smoke-induced inflammation was a logical next step for this research.

Chapter 4 - RELB expression and suppression of cigarette smoke-induced COX-2 expression

4.1 Results

4.1.1 RELB expression is decreased in smokers and COPD patients:

RELB is an essential part of the non-canonical NF-κβ pathway and contributes to the regulation of inflammation[149,157]. Lung inflammation as a result of smoking is a prominent feature of COPD pathology, and has been shown to involve a host of inflammatory mediators[33,186], including COX-2[186]. Our data (Chapter 3) highlight the importance of the AHR as an attenuator of COX-2 protein expression. The ability of the AHR to attenuate cigarette smoke-induced COX-2 may be due to the preservation of RELB protein expression[142]. However, there is no information on RELB in COPD. Therefore, to understand if RELB is connected to the pathology of COPD, we first examined the basal expression of RELB in Control, Smoker and COPD subject-derived lung fibroblasts by PCR and western blot. The expression of RELB is shown in Figure 4.1. Both smokers- and COPD-derived lung fibroblasts exhibit significantly lower RELB protein (Figure 4.1A and B) and mRNA (Figure 4.1C) expression in comparison to the control lung fibroblasts. There was no significant difference in RELB protein or mRNA expression between Smoking and COPD-derived lung fibroblasts.

4.1.2 Exposure to 2% CSE results in loss of RELB protein:

As smoking may be a contributing factor to the expression of RELB (Figure 4.1), and knowing that RELB is degraded in a proteasome-dependant manner[211], we examined if

cigarette smoke exposure contributes to a reduction in RelB. Control subject-derived fibroblasts (chosen due to their relative expression of RELB) were treated with 2% CSE for 3, 6 and 24 hours and the RELB expression was assessed. Figure 4.2A demonstrates that treatment with CSE resulted in the progressive loss of RELB protein. There was an approximate 60% decrease in RELB protein after 6 hours, and 80% of RELB protein was lost after 24 hours of 2% CSE treatment. *Relb* mRNA expression (Figure 4.2B) decreased after 6 hours of treatment with 2% CSE, and then recovered by 24 hours. These data indicate that CSE promotes a reduction of RELB protein, but not *Relb* mRNA.

4.1.3 Cox-2 mRNA is increased in COPD derived lung fibroblasts over Control in response to CSE:

As RELB is a key repressor of inflammation[110,145,152,157], we hypothesized that low RELB protein in COPD cells may contribute to an increase in *Cox-2* mRNA. In Control- and COPD subject derived fibroblasts treated with 2% CSE, significant induction of *Cox-2* mRNA was only observed in COPD-derived fibroblasts (Figure 4.3A). No significant increase in COX-2 protein as a result of 2% CSE treatment was seen, although trended towards significance at 6 hours (Figure 4.3B).

4.1.4 RELB suppresses induction of Cox-2 mRNA:

siRNA knockdown of RELB in Control fibroblasts was used to confirm if the difference in *Cox-2* mRNA expression was due to the absence of RELB protein. siRNA knockdown of RELB resulted in an approximate 40% reduction in expression compared to control

siRNA (Figure 4.4A). RELB siRNA knockdown lung fibroblasts were then treated with 2% CSE for 6 hours. *Cox*-2 mRNA induction after siRNA knockdown and 2% CSE treatment was also significantly higher compared to both siRNA knockdown and control fibroblasts treated with media alone (Figure 4.4B). These data confirm that RELB expression suppresses the production of *Cox-2* mRNA in lung fibroblasts exposed to CSE, and indicate the difference in *Cox-2* mRNA expression between Control and COPD subject derived fibroblasts treated with 2% CSE may be due to lower expression of RELB.

4.1.5 miR-146a expression is not different in Control, Smoking and COPD derived fibroblasts:

The mechanism by which RelB controls COX-2 protein expression is not known, but may involve miR-146a. miR-146a targets *Cox-2* mRNA for degradation and has been implicated in the pathogenesis of COPD[93]. Here, COPD lung fibroblasts produce less miR-146a compared to fibroblasts derived from smokers when challenged with pro-inflammatory cytokines[93]. However, whether miR-146a is altered by cigarette smoke or whether RelB controls miR-146a is not known. Therefore, we first examined the baseline expression of miR-146a in Control, Smoker and COPD subjects. Baseline expression of miR-146a was however not significantly different between Control, Smoker or COPD patient derived lung fibroblasts (Figure 4.5)

4.1.6 miR-146a induction by CSE or IL-1β is abrogated in COPD fibroblasts:

miR-146a induction in response to inflammatory stimuli has been shown to be regulated at least in part by the classic NF-κβ pathway due to κβ sites present within the miR-146a promoter[117]. As IL-1β has been shown to activate inflammatory NF-κβ gene transcription[212] and to be elevated in COPD[2], we therefore treated Control and COPD subject derived fibroblasts with IL-1β to determine if there was a difference in induction of miR-146a. Figure 4.6A demonstrates that in response to IL-1β, Control fibroblasts upregulate the expression of miR-146a whereas COPD-derived fibroblasts do not. Next, we wanted to determine if the difference in miR-146a between Control and COPD fibroblasts also occurred upon exposure to CSE. Two percent CSE upregulated miR-146a in Control, but not COPD, fibroblasts (Figure 4.6B). Together, these results suggest that the COPD patient-derived lung fibroblasts lack the capacity to upregulate miR-146a expression.

4.1.7 miR-146a induction in response to IL-1β, not CSE, requires RELB expression:

To now determine if RELB expression in Control lung fibroblasts is necessary for miR-146a expression in response to IL-1β or CSE, Control patient-derived fibroblasts were transfected with siRNA against RELB. Subsequent to RELB knockdown, the capacity of Control subject derived lung fibroblasts to increase expression of miR-146a in response to IL-1β was significantly reduced (Figure 4.7A). When treated with 2% CSE, no significant difference in induction of miR-146a between control siRNA or RELB siRNA was seen. Additionally, 6 hour treatment with 2% CSE did not induce a significant

change in miR-146a relative to media treated fibroblasts. Therefore, miR-146a regulation by RELB may be stimulus-specific.

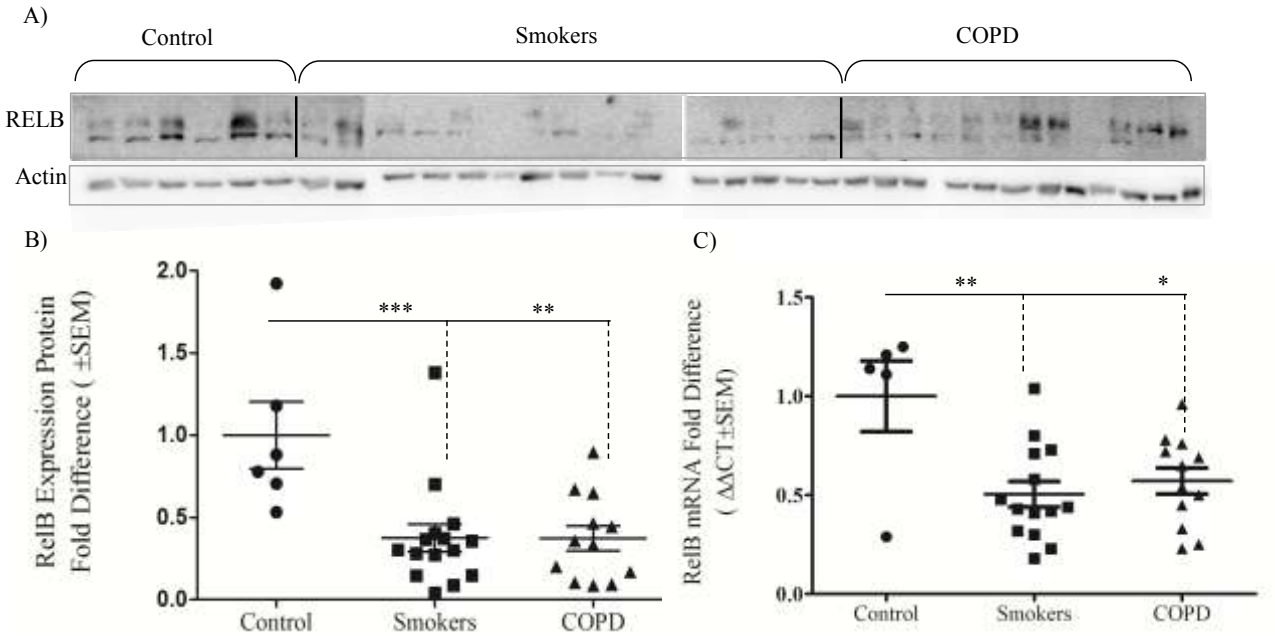

Figure 4.1. RELB Expression is Decreased in Smoking and COPD Patient-Derived Fibroblasts A) Western blot for Control, Smokers and COPD derived lung fibroblasts. B) Densitometry from A)- there was a significant decrease in RELB protein expression in lung fibroblasts derived from the lungs of Smokers as well as COPD subjects (** $p \leq 0.01$, ***$p \leq 0.001$ compared to fibroblasts from Control subjects. C) RELB mRNA expression was significantly decreased in smoker and COPD derived fibroblasts as compared to that of control patient derived fibroblasts (** $p \leq 0.01$, * $p \leq 0.05$). Each symbol represents fibroblasts derived from a different individual.

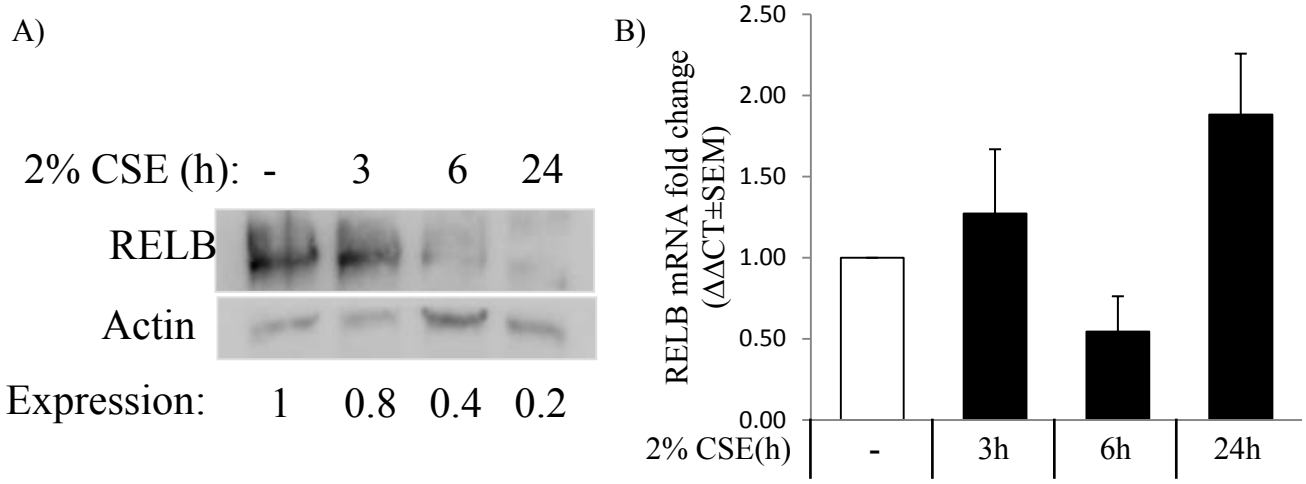

Figure 4.2. 2% CSE treatment reduces RELB protein expression, but does not significantly change mRNA expression. A) There was a progressive decrease in the expression of RELB over time in Control patient derived lung fibroblasts after exposure to 2% CSE. A representative western blot is shown. B) *RELB* mRNA expression in control lung fibroblasts (n=3) treated with 2% CSE after 3, 6 and 24 hours. No significant differences were found in mRNA expression.

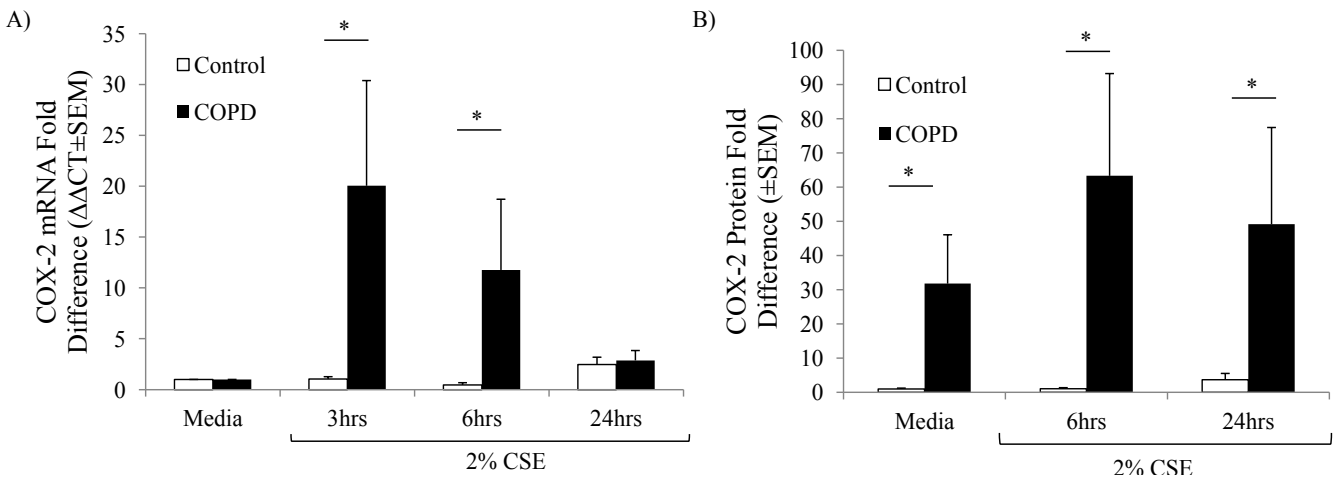

Figure 4.3. COX-2 mRNA expression is elevated in response to CSE in COPD subject fibroblasts. A) Treatment of Control and COPD patient derived lung fibroblasts with 2% CSE for 3, 6 and 24 hours significantly increased COX-2 mRNA in COPD derived, but not Control fibroblasts, as determined by qRT-PCR (3hr & 6hr, $p \leq 0.05$). B) COX-2 Protein expression after 2% CSE treatment in Control and COPD patient derived lung fibroblasts for 6 and 24 hours. CSE treatment in COPD derived lung fibroblasts resulted in significantly greater COX-2 expression than Control (6hr & 24hr, $p \leq 0.01$).

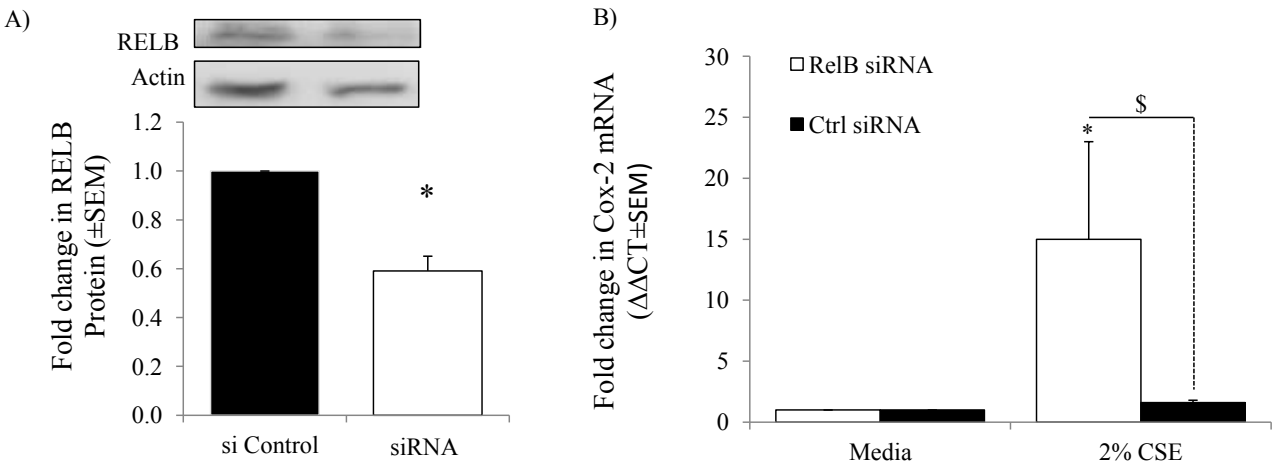

Figure 4.4 Control derived lung fibroblasts produce more *COX-2* mRNA when RELB expression is reduced. A) RELB siRNA transfected into Control patient derived lung fibroblasts resulted in an approximate 40% reduction in RELB expression. B) RELB siRNA transfected lung fibroblasts subsequently treated with 2% CSE for 6 hours increased expression of COX-2 mRNA as compared to control siRNA transfection.($p \leq 0.05$).

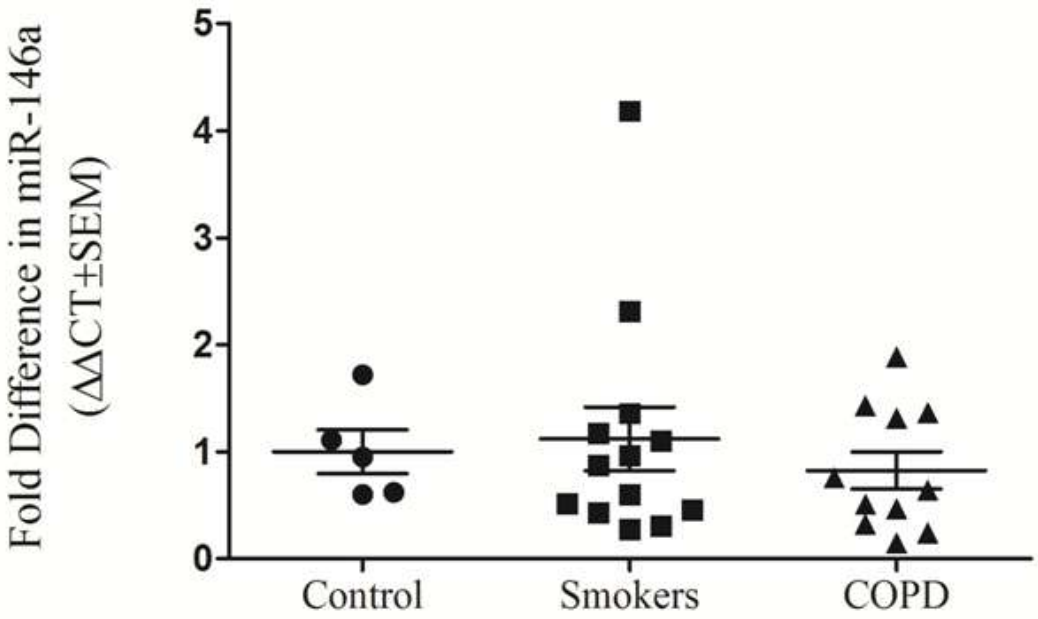

Figure 4.5. Basal miR-146a expression is not significantly different in human lung fibroblasts from Control, Smokers or COPD patients. There was no significant difference in the baseline expression of miR-146a between fibroblasts derived from Control, Smokers or COPD patients, as determined by RT-qPCR. Each point represents a different individual patient.

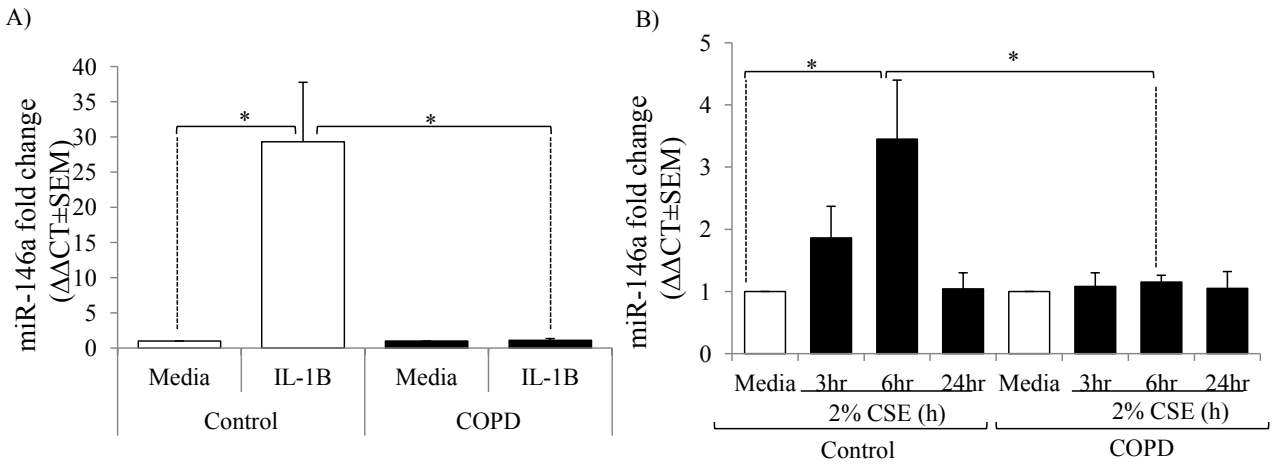

Figure 4.6. miR-146a expression is increased by treatment with IL-1B or 2% CSE in control fibroblasts only. A) Significant induction of miR-146a after treatment with IL-1B for 6 hours (10ng/ml) was seen in control patient derived fibroblasts as compared to media treatment ($p \leq 0.05$). COPD derived fibroblasts did not increase miR-146a expression after IL-1B treatment, as compared to the control fibroblasts ($p \leq 0.05$). B) Treatment with 2% cigarette smoke significantly increased the expression of miR-146a in control patient derived fibroblasts after 6 hours of treatment ($p \leq 0.05$). This effect was not observed in the COPD derived fibroblasts.

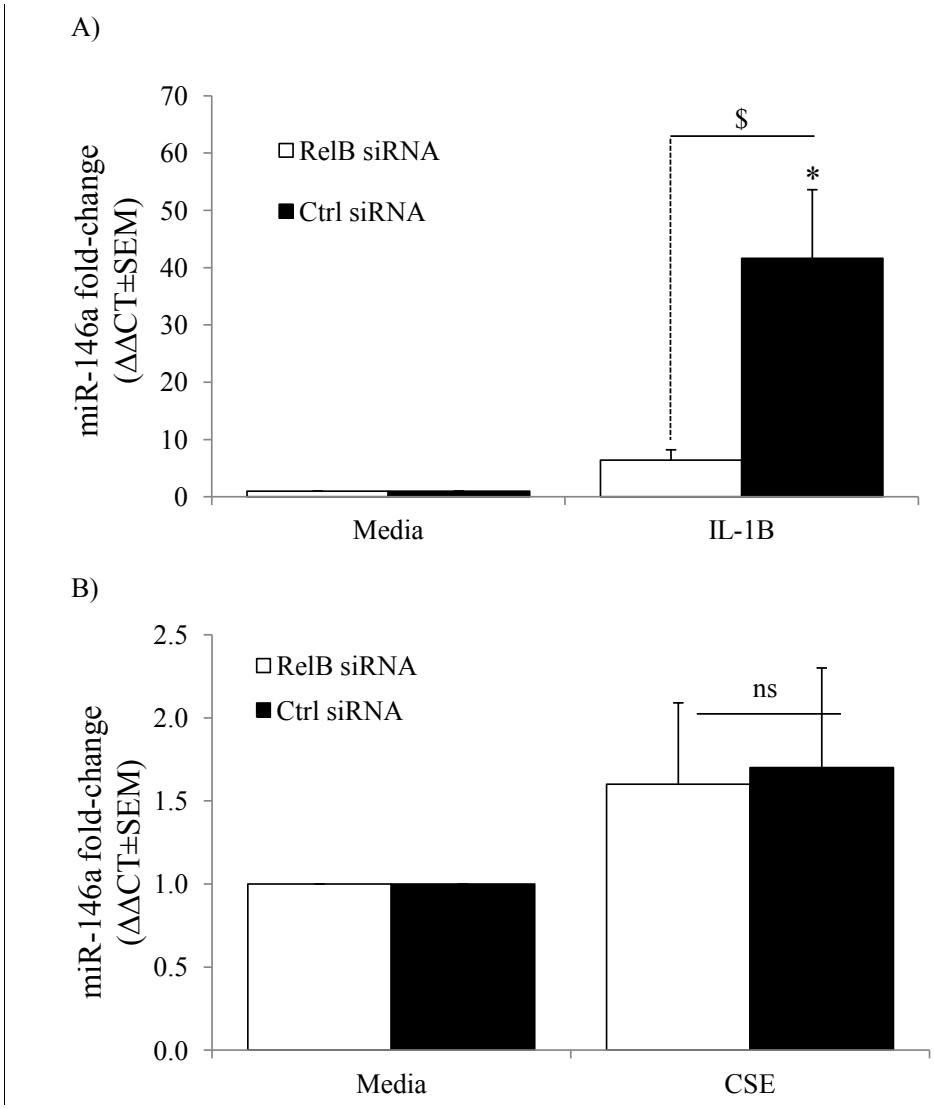

Figure 4.7. RELB knockdown decreases control fibroblast's expression of miR-146a. RELB siRNA knockdown in control patient derived human lung fibroblasts was accomplished with 60 nM of siRNA against RELB, or Control (scramble). 6 hours past transfection, Serum free media was added for 18 hours. 24 hours past the initial transfection, treatment with IL-1B (10ng/ml) or 2% CSE was added for 6 hours before harvesting for RNA. Subsequent qRT-PCR analysis revealed that siRNA knockdown prevented IL-1B from inducing miR-146a expression. 2% CSE treatment did not increase the expression of miR-146a significantly in either control or knockdown. (N=3)

4.2 Discussion

RELB has been linked to repression of smoke-induced COX-2 production in mouse lung fibroblasts[158] and IL-1β-induced inflammation in human lung fibroblasts[110], but no information existed concerning the expression or regulation of RELB in COPD subjects. How RELB expression changes with disease is not well understood but *Relb*-deficient mice exhibit multi-organ inflammation, constitutive expression of inflammatory cytokines (IL-1, IL-2, TNFα) and impaired immune cell differentiation[157,213]. Lungs of *Relb*-deficient mice are particularly affected, in which widespread lymphoid infiltration of granulocytes and macrophages has been observed[157], similar to that seen in COPD. Despite this, the relationship of RELB expression, smoke exposure and COPD has yet to be evaluated. COX-2 expression is elevated in COPD subjects[39,93], and is upstream of inflammatory mediators such as PGE_2, IL-8, IL-6 and TNFα[33,214]. Moreover, inhibition of COX-2 halts the progression of emphysema in rat models[100]. Therefore, this study aimed to characterize RELB expression in Control, Smoker and COPD lung fibroblasts and provide a link to the increased COX-2 in COPD subjects. We also explored the mechanism through which RELB regulates COX-2 expression in response to CSE by evaluating the expression of miR-146a We report the novel findings that basal RELB expression is decreased in Smoker and COPD subjects, and RELB expression is important for repression of *COX-2* mRNA. Additionally, although miR-146a does not appear to be regulated by RELB, it is differentially expressed in Control and COPD subject lung fibroblast in response to smoke.

The expression of RELB in Control, Smoker, and COPD subjects was previously unknown. We report that in comparison to Control subjects, Smoker and COPD subjects

express reduced RELB protein and mRNA (Figure 4.1). It is not yet clear what regulates expression of *Relb* at the transcriptional level, but may include epigenetic remodelling. Cigarette smoke results in aberrant methylation of the genome[170,215], which may be reversible (depending on the gene locus) after a subject quits smoking[216]. Hypermethylation, investigated either at specific loci or genome wide[169], inversely correlate to the expression of methylated genes[217]. Chronic smoke exposure may progressively silence the *Relb* gene via methylation[177]. One study indicated that acrolein, an environmental contaminant and component of cigarette smoke, contributes to the remodeling of chromatin via the deaceytlation of free histones[171]. Histone deaceytlation is associated with repression of genes, as deacetylated histones are able to more closely sequester DNA from transcription factors[218]. Acrolein binds preferentially to methylated CpG DNA sites[172] contributing to DNA damage and gene silencing. Cigarette smoke-induced deacytlation of histones, and gene methylation may therefore render *Relb* inaccessible for transcription, thereby reducing mRNA levels. Taken together, it is plausible that chronic smoke exposure reduces the expression of *Relb* mRNA over time via gene methylation and epigenetic remodelling of chromatin.

The reduced RELB protein expression in Smoker and COPD subjects follows the reduction in *RelB* mRNA expression. RELB protein is also degraded in response to acute CSE exposure in a proteasome-mediated manner[152,211] and the CSE mediated degradation is supported by our own data (Figure 4.2). Thus, proteasomeal degradation of RELB in response to CSE may be one component in the overall loss of RELB expression in Smoker and COPD groups. Interestingly, acute CSE exposure has also been reported to reduce the capacity of the proteasome to degrade proteins[219]. This is of importance, as

regulation through protein degradation occurs at three stages of the RELB pathway: NIK accumulation; p100 processing; and RELB proteosomal degradation after activation[149,220]. When NIK degradation is impaired, NIK accumulates and becomes constitutively activated[150,221] and may contribute to altered RELB degradation. Degradation of RELB despite proteasomal inhibition by smoke exposure necessitates another method of degradation such as the autophagic pathway. The oxidative stress and protein carbonylation characteristic of cigarette smoke[222,223] may account for additional degradation via the induction of autophagic processes[224]. Additional mechanisms may involve mucosa-associated lymphoid tissue lymphoma translocation protein 1(MALT1), which cleaves RELB, thereby preventing RELB mediated repression of the classical NF-κβ pathway[225]. MALT1 cleavage of RELB results in a 55kDa product[225], and is observed in our model as the lower of the two bands (Figure 4.1A).

Reduced RELB expression contributes to increased expression of *COX-2* mRNA in COPD subject-derived lung fibroblasts in response to inflammatory challenge. The baseline difference in COX-2 protein seen between Control, Smoker and COPD subject-derived fibroblasts (Figure 3.2A) was unaccompanied by a basal difference in *COX-2* mRNA despite a clear difference in RELB expression, suggesting RELB does not control basal COX-2 expression. Likewise, there was no difference in *COX-2* mRNA expression between Control or COPD subject-derived fibroblasts when treated with media alone (Figure 3.2). The twenty-fold difference in *COX-2* mRNA expression seen after CSE treatment between COPD and Control derived lung fibroblasts (Figure 4.3A) suggested RELB expression is necessary for the repression of *COX-2* induced in response to smoke. We were able to confirm RELB suppression of COX-2 mRNA production via siRNA

knockdown of RELB (Figure 4.4). Repression is likely occurring at the promoter level as the *COX-2* gene contains multiple κβ sites at which RELB can bind[102,110]. RELB has also been reported to repress genes of inflammatory mediators such as TNFα, via binding to κβ in direct competition with p65/p50 complexes[156,226], or as a transcriptionally inactive complex containing either p100[149], or p52[156]. A ChIP assay could be used to determine if *COX-2* is being repressed via promoter binding, as well if there are multiple subunits involved.

We next hypothesized that COX-2 protein expression was being controlled by post-transcriptional regulation as basal COX-2 protein expression did not correlate to basal *COX-2* mRNA expression in COPD subject derived fibroblasts (Figure 3.2A). Recent research has begun to investigate the role of miRNAs as components involved in the post-transcriptional control of protein expressin[116,117,190,227]. In particular, miR-146a is reduced in COPD[93,228]. miR-146a targets COX-2 mRNA for degradation[93]. Surprisingly, there was no basal difference in miR-146a expression between Control, Smoker or COPD subject derived fibroblasts (Figure 4.5), suggesting that the basal difference in COX-2 protein expression is not mediated by miR-146a. However, McMillan *et. al.*[110] demonstrated that RELB is an important regulator of miR-146a in response to inflammatory challenge by IL-1β. We were able to confirm increased miR-146a expression in Control, but not COPD subject derived fibroblasts, in response to IL-1β (Figure 4.6A) and CSE (Figure 4.6B). We proposed that the difference in miR-146a expression between Control and COPD was due to the loss of RELB in COPD subjects. Knockdown of RELB in Control subject derived fibroblasts confirmed RELB is key to the regulation of miR-146a after treatment with IL-1β (Figure 4.7A), as induction of

miR-146a was seen in siRNA control, but not siRNA RELB cells. However, there was no difference in miR-146a expression after CSE treatment between control or RELB siRNA (Figure 4.7B). It is possible that the transfection system affected the induction of miR-146a. Also, 40% knockdown of RELB protein may not be sufficient to reveal the relationship of miR-146a to RELB after CSE exposure. An additional explanation is that miR-146a regulation in response to smoke is not mediated by RELB, and that there is an alternate pathway through which CSE induces miR-146a. This would indicate that RELB repression of *COX-2* mRNA production is a complementary pathway to miR-146a regulation of COX-2 protein. NF-κβ induction of miR-146a in response to smoke is a potential alternate mechanism.

Collectively, our results illuminate the relationship between RELB and inflammation in COPD, but also open the field to further research on the exact mechanistic regulation of both RELB expression and miR-146a in response to smoke. We have shown that RELB expression is necessary to prevent exaggerated COX-2 mediated inflammation, and that this is lost in COPD subjects. RELB therefore represents an interesting opportunity for future longitudinal studies of COPD to determine if RELB loss is a symptom of COPD, or a causative factor in the progression of the disease. RELB represents an interesting target for future study, and for pharmaceutical agents to treat COPD.

Chapter 5 - Future Directions: Interaction of the AHR and RELB

5.1 AHR and RELB interactions

The previous chapters have concentrated on the expression and function of the AHR or RELB, and how each in their own right, contributes to smoke-induced COX-2 expression. We have shown that both AHR and RELB expression is decreased in fibroblasts from COPD subjects, and presented arguments as to how the loss of AHR or RELB increase expression of COX-2 in response to CSE. Herein, we wish to address how the AHR and RELB pathways intersect and offer preliminary findings concerning their physical association. Until recently, very little was known regarding interaction between AHR and RELB. In 2011 McMillan et al. demonstrated that the AHR mediated repression of COX-2 expression after smoke exposure via RELB[158]. This supports the 2008 finding from Baglole et al. that fibroblasts from $AHR^{-/-}$ mice rapidly lost RELB expression after smoke[106]. Vogel et al., utilizing a human monocytic cell line, demonstrated that AHR and RELB can physically associate, and that a AHR:RELB complex may bind to gene promoters containing an XRE, a κβ site, or containing a previously unknown binding sequence (5'- agatgagggtgcataag -3') termed the RELB/AHRE[159,229]. These results are interesting in light of our presented findings, suggesting that crosstalk between the pathways (AHR and RELB) may offer a mechanistic explanation for AHR- and RELB-dependant suppression of *Cox-2* expression.

Questions concerning the AHR and RELB pathways crosstalk remain in relation to their ability to suppress inflammatory responses in the lung. Do the AHR and RELB physically associate? Is this association altered by exposure to respiratory toxicants such

as cigarette smoke? Does disruption of AHR/RELB interaction promote proteolytic degradation of RELB in response to cigarette smoke? To begin addressing these questions, we utilized U937 cells to evaluate the physical association between AHR and RELB. Our preliminary data are consistent with the physical association between AHR and RELB in U937 macrophages reported by Vogel et al., (Figure 6.1). Moreover, these data also indicate that exposure to CSE may increase the association of AHR and RELB. This suggests the response to CSE is different compared to previous reports utilizing classic AHR ligands, as TCDD did not affect the AHR/RELB association[159]. It is unclear if the association of AHR and RELB in U937 cells extends to different cell types, and future experiments to test the effect of CSE on AHR/RELB physical association in lung fibroblasts will determine if CSE induced physical of AHR and RELB occurs here as well. Furthermore, it is unknown if AHR and RELB associate only within nucleus as reported[210], or within the cytoplasm as well. Cytoplasmic association of RELB and AHR may indicate a chronological order to the association or a regulatory role independent of classic ligand binding. Future co-immunoprecipitation of AHR and RELB in nuclear and cytoplasmic fractions could be performed to determine this.

It is also unclear if the association of AHR and RELB is dependent on AHR activity. AHR inhibition may disrupt AHR and RELB association. Disruption of the AHR/RELB association may increase the amount of RELB available to bind to p100, or to other NF-κβ family members, shifting the balance from gene repression to activation. Disruption of the AHR/RELB complex may also make RELB vulnerable to degradation, possibly explaining the loss of RELB expression in $AHR^{-/-}$ mice seen previously[106]. It is unclear how disruption of this complex may affect the regulation or expression of the

AHR. It is possible that disruption of AHR/RELB complexes may increase classical AHR transcription, as AHR is made available to bind classic XREs. It is unknown if RELB-deficient mice express more classical AHR dependent genes, like *Cyp1b1* for example. Long term dysregulation of AHR/RELB complexes may also increase proteasome mediated degradation of AHR and RELB. Disruption of the AHR/RELB complex may make both AHR and RELB vulnerable to degradation in the cytoplasm. Increased physical association caused by CSE may protect AHR/RELB against degradation in the cytoplasm, although long term CSE exposure is known also result in degradation of both AHR and RELB subsequent to activation[152,230].

Ligand-activated RELB is known to form transcriptionally inactive complexes with RELA[231], which may present a mechanism by which AHR/RELB complexes may direct specific gene repression. This mechanism may involve recruitment of RELA, with AHR acting as a co-factor to direct the RELB/RELA repressive complex to specific promoter sites. RELB both suppresses and activates genes[232], suggesting it is also possible AHR may direct RELB to specific genes in response to cigarette smoke exposure in order to elicit transcription. AHR/RELB specific transcription is a potential mechanism that would explain the difference in miR-146a expression between Control and COPD subject fibroblasts (Figure 4.6). AHR/RELB complexes in Control lung fibroblasts may be recruited to miR-146a promoters in response to cigarette smoke, a mechanism that would be lost alongside decreased AHR and RELB protein in COPD fibroblasts. Other factors, such as chaperone proteins are critical to both the AHR and non-canonical NF-κβ pathways[149,233], and it is not known if they are necessary for AHR:RELB complexes. Co-immunopreciptiation of HSP90, p100 or p52 may help

determine the relative importance of these additional proteins in the function or expression of the AHR:RELB association. Figure 5.2 is a schematic of what is known currently about the intersection of non-canonical NF-κβ and AHR pathways, highlighting areas for future investigation. Experiments to further investigate the crosstalk between RELB and AHR are ongoing, and should determine the relevance of this association in the context of the lung.

Regulation of the mechanisms downstream of AHR and RELB that control *Cox-2* stability we have previously discussed (AHR mediated HUR nucleo-cytoplasmic shuttling, and RELB mediated miR-146a transcription) may also be interrelated. To date, little has been shown to link HUR directly with RELB or miR-146a, but new research has shown HUR mRNA may be a target for miR-146a[234]. miR-146a expression may fine tune HUR production, and suggests at the importance of this pathway in the context of lung inflammation. Furthermore, HUR has been shown to be capable of repressing the activity of other miRNAs through promoting dissociation of the RISC complex[235,236]. Interestingly, miRNAs shown to be repressed by HUR include miR-16, which is capable of targeting *Cox-2*[235]. This strengthens the notion of a co-regulatory relationship between AHR and RELB, as well as the pathways they regulate. This data also supports the evidence we have provided on the effects of COPD related loss of AHR and RELB. Future experiments to determine if HUR is capable of miRNA repression in the lung, and specifically miR-146a, may provide another link between AHR and RELB.

This thesis research has explored the AHR and RELB in the context of COPD in pulmonary fibroblasts, including the ability of the AHR and RELB to regulate COX-2 expression. We also provided mechanistic insight into the molecular regulation by

demonstrating first that COX-2 is repressed through AHR-dependent regulation of HUR. We further demonstrate that RELB and miR-146a are important in the regulation of COX-2 in response to cigarette smoke. Although increased AHR/RELB physical association in response to cigarette smoke remains largely unclear, we have provided a direction which could become a focus of future experiments. It is evident that both the AHR and RELB are important in lung inflammation, worthy of additional investigation in the context of COPD as potential targets for the therapeutic management of COPD.

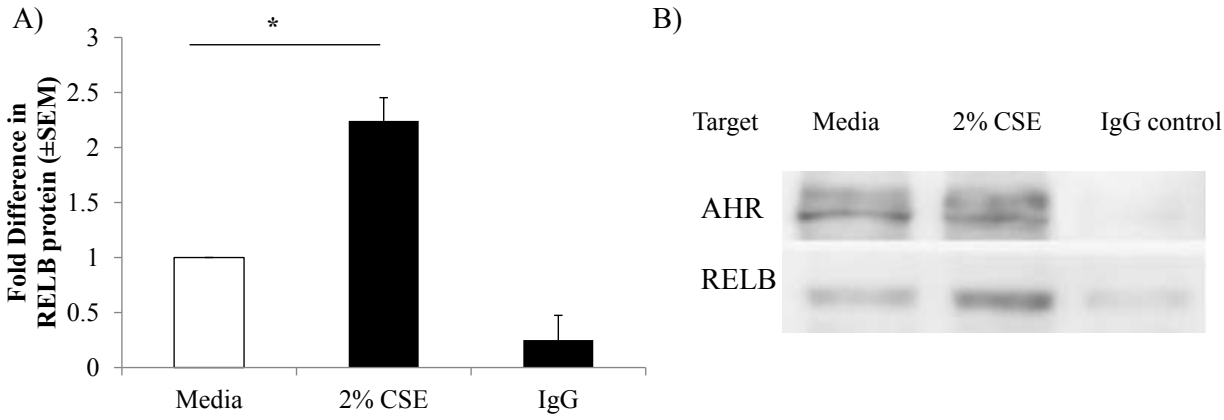

Figure 5.1. Cigarette smoke treatment increases the physical association of AHR and RELB A) U937 Macrophage cells treated with 2% CSE for 6 hours and then lysed. Whole cell lysates that were co-immunoprecipitated for AHR showed an approximate two-fold increase in the amount of RELB in the immuoprecipitated fraction. AHR and RELB (N=2). B) Representative western blot for the co-immunoprecipitation of AHR and RELB. No appreciable AHR was detected in the IgG control fraction.

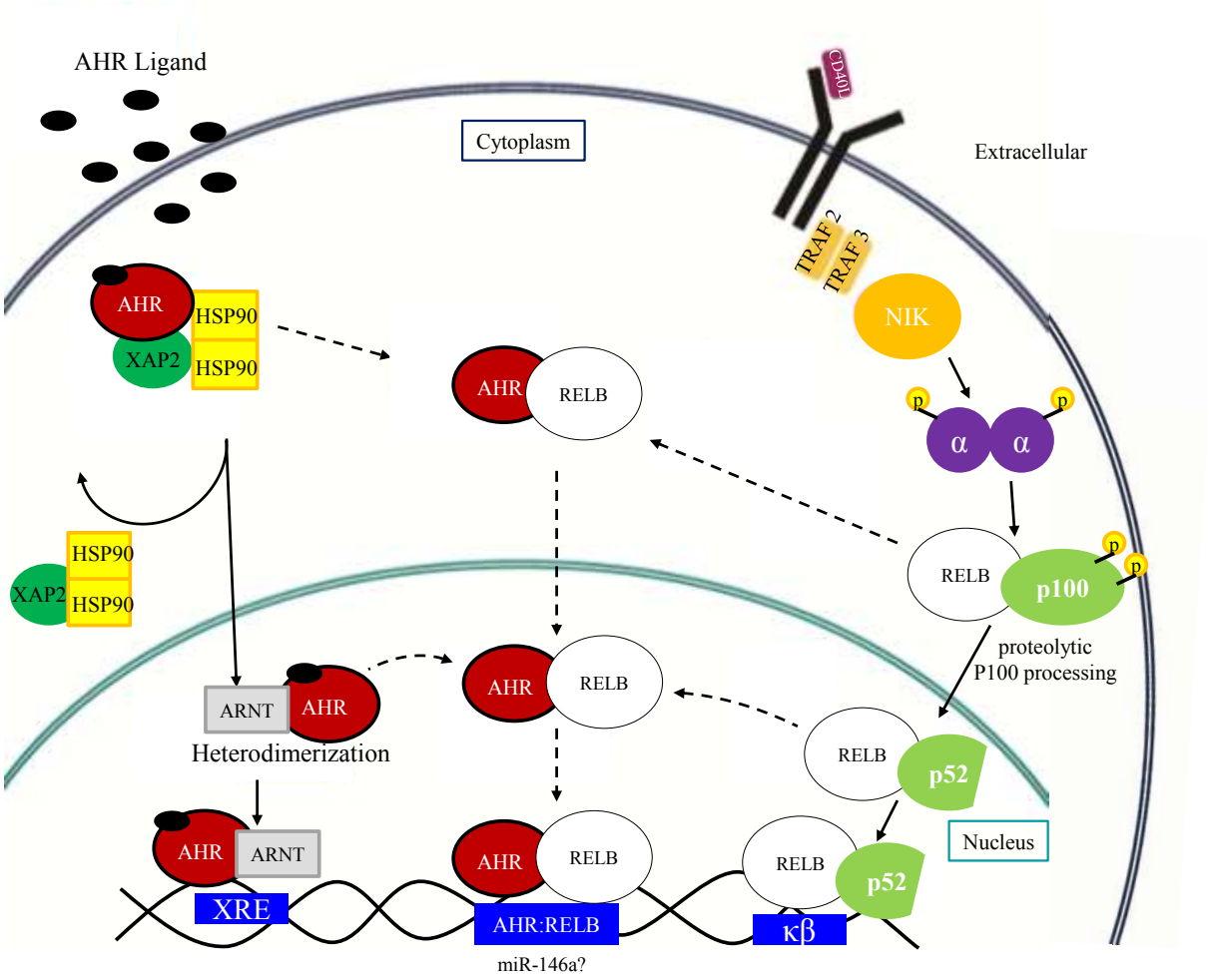

Figure 5.2 The AHR and RELB pathways, with potential areas of crosstalk. Dashed arrows indicate putative pathway steps for future research.

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