Human antigen R (HuR) regulates Cigarette Smoke-induced inflammation: Implications in COPD

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LIST OF ABBREVIATIONS

Abbreviation	*Meaning*
α1AT	Alpha 1-anti-trypsin
AhR	Aryl hydrocarbon receptor
ATP	Adenosine triphosphate
AUF1	AU-binding factor 1
BAL	Bronchoalveolar lavage
cAMP	Cyclic adenosine monophosphate
CARM1	Coactivator-associated arginine methyltransferase 1
CCL2	Monocyte chemoattractant protein 1
COPD	Chronic Obstructive Pulmonary Disease
COX-2	Cyclooxygenase-2
CRM1	Chromosome region maintenance 1
CSE	Cigarette smoke extract
CS	Cigarette smoke
CUGBP2	CUG triplet repeat RNA-binding protein 2
ECM	Extracellular matrix
ELAV	Embryonic lethal abnormal vision
ELAVL-1	Embryonic lethal abnormal vision-like protein 1
FADD	Fas associated via death domain
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GR	Glucocorticoid receptors
hnRNP D	Heterogeneous nuclear ribonucleoprotein D
HNS	HuR nuclear shuttling domain
HuR	Human antigen R
ICAM-1	Intercellular adhesion molecule-1
ICS	Inhaled corticosteroids
IL	Interleukin
LABA	Long-acting β-agonists
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NE	Neutrophil Elastase
NF-κB	Nuclear Factor Kappa B
PDCD4	Programmed cell death 4
PG	Prostaglandin
PK	Protein kinase
RBP	RNA-binding protein
RBM3	RNA binding motif protein 3
RRM	RNA recognition motif
SGs	Stress granules
SLPI	Secretory leukoprotease inhibitor
TGF-β	Transforming growth factor β
TIA-1	T-cell Intracellular Antigen 1
TNF-α	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor

ABSTRACT

Chronic Obstructive Pulmonary Disease (COPD) is an incurable and prevalent respiratory disorder that is characterized by chronic inflammation. This inflammation is typified by an increase in inflammatory cells and mediators such as interleukin-8 (IL-8) and cyclooxygenase-2 (COX-2). COPD is primarily caused by cigarette smoke (CS) and other noxious particles (*e.g*. air pollution). Cigarette smoke may contribute to lung inflammation via human antigen R (HuR), a ubiquitously-expressed RNA-binding protein that regulates the stability of mRNA encoding proteins that are associated with inflammation; this would indirectly increase expression by facilitating translation of the mRNA. In order for HuR to stabilize target mRNA, HuR translocate from the nucleus to the cytoplasm. However, the expression and localization of HuR in COPD or its molecular regulation by cigarette smoke is not known. Therefore, we hypothesize that cytoplasmic translocation of HuR contributes to inflammatory protein production in response to cigarette smoke, including those that are increased in COPD. The aims of this study are (1) to evaluate the expression and localization of HuR in human lung tissue and structural cells from Normal (never-smokers without COPD), Smokers (without COPD) and COPD subjects and (2) to assess if HuR regulates cigarette smoke-induced inflammatory protein production. First, by using multiplex Immunohistochemistry (mIHC), we found that there was more cytoplasmic localization of HuR in lung tissue from Smokers and COPD subjects compared to Normal subjects, where HuR was predominantly localized in the nucleus. There was also more cytoplasmic HuR in macrophages from Smoker and COPD lung tissue. *In vitro,* in primary human lung fibroblasts (HLF), there was a slight increase in total HuR protein in Normal and COPD HLF after exposure to 2% cigarette smoke extract (CSE), an *in vitro* surrogate for cigarette smoke exposure. After 4 hours of CSE exposure, there was an increase in cytoplasmic HuR. Surprisingly, the protein and mRNA levels of COX-2 and IL-8 were significantly higher in

siHuR-transfected cells exposed to 2% CSE for 24 hours compared to siCtrl-transfected cells. Finally, Knock-down of HuR did not affect the decay of *cox-2* and *Il-8* mRNA in response to 2% CSE. This study is the first that investigate the role of HuR in regulating cigarette smoke-induced inflammation which could provide the basis for the development of new target therapy for diseases such as COPD.

RÉSUMÉ

La bronchopneumopathie chronique obstructive (BPCO) est une maladie respiratoire grave caractérisée par une obstruction lente et progressive des voies aériennes entrainant une gêne respiratoire. La BPCO débute par une inflammation chronique dans les poumons due à une augmentation de nombre des cellules immunitaires et de l'expression de cytokines inflammatoires (ex : l'interleukine-8 (IL-8) et la cyclooxygénase-2 (COX-2)). Dans cette étude nous avons voulu étudier si une protéine nommée antigène humain R (HuR), connue d'avoir un rôle inflammatoire, est impliquée dans le développement de la BPCO. HuR est une protéine qui se lie à l'ARN messager (ARNm) et augment sa stabilité et sa traduction en protéines. Parmi ces ARNm régulés par HuR, on retrouve ceux qui codent pour des cytokines inflammatoires (ex : IL-8 et COX2). Ceci augmenterait donc indirectement le taux de ces cytokines en facilitant la traduction de l'ARNm. Pour que HuR stabilise l'ARNm cible, il doit transloquer du noyau vers le cytoplasme. Bien que HuR est connu d'être impliqué dans plusieurs maladies inflammatoires, sa régulation par la fumée de cigarettes ainsi que son implication dans le développement de la BPCO ne sont pas connues. Par conséquent, on a mis l'hypothèse que la translocation cytoplasmique de HuR suite à l'activation par la fumée des cigarettes contribue à une augmentation de la production de protéines inflammatoires. Le premier objectif de cette étude est donc d'évaluer l'expression et la localisation de HuR dans les tissus pulmonaires humains et les dans les cellules structurelles venant des sujets, des fumeurs et des sujets atteints de BPCO. Deuxième objectif c'est de déterminer si HuR Régule la production de protéines inflammatoires induites par la fumée de cigarette. Tout d'abord, en utilisant l'immunohistochimie multiplex (mIHC), nous avons observé qu'il y avait plus de localisation cytoplasmique de HuR dans le tissu pulmonaire chez les fumeurs et les sujets atteints de BPCO par rapport aux sujets sains. Chez ces derniers, HuR était principalement localisé dans le noyau. En outre, et d'une manière intéressante, les macrophages chez les fumeurs et les sujets atteints

avait d'HuR dans les leur cytoplasme beaucoup plus chez les sujets sains. Dans un deuxième temps, nous avons démontré que le traitement des fibroblastes primaires pulmonaires humains (HLF), augmenterait le taux protéique totale d'HuR et sa translocation après exposition à 2% d'extrait de fumée de cigarette. Enfin, et afin de plus étudier le rôle de HuR dans l'inflammation induite par la fumée de cigarette nous avons utilisé un petit ARN interférent contre HuR. Cependant, et contrairement à ce qui est attendu, nous avons observé que lorsque HuR a été réduit avec un ARN interférent contre HuR, le taux de l'ARNm et de protéines de COX-2 et d'IL-8, induite avec l'extrait de fumée de cigarette (2%), était significativement plus élevés par rapport aux cellules transfectées par un ARN interférent control. Enfin, nous avons observé que dans les cellules transfectées avec un ARN interférent contre HuR et traité avec l'extrait de fumée de cigarettes (2%), la stabilité de l'ARNm de cox-2 et Il-8 était comparable par rapport aux cellules contrôles. L'ensemble de ces données suggèrent que HuR pourrait réguler l'expression de COX-2 et de l'IL-8 en réponse à la fumée de cigarettes sans affecter la stabilité de leurs ARNm.

Notre étude est la première qui essaye de comprendre comment HuR régule l'inflammation induite par la fumée de cigarettes. Ceci donc peut favoriser le développement des nouvelles stratégies ciblant HuR pour le traitement des maladies causées par le tabagisme comme la BPCO

CHAPTER 1: INTRODUCTION

1.1 COPD: definition, disease characteristics

Chronic Obstructive Pulmonary Disease (COPD) is an important public health problem, as it is a leading cause of chronic morbidity and mortality worldwide [1]. The World Health Organization (WHO) in 2012 listed COPD as the third leading cause of death [2], with its prevalence expected to increase in coming decades. During the period from 1970 to 2002, mortality rates due to cardiac diseases and stroke decreased, but that of COPD doubled [3]. The definition of COPD- as given by the Global Initiative for Chronic Obstructive Lung Disease (GOLD)- is a lung disease characterized by progressive and irreversible airflow limitation, which is usually associated with an abnormal inflammatory response in the airways and lungs to noxious particles or gases. This chronic airflow limitation encompasses both emphysema, which is the irreversible destruction of alveolar sacs, and chronic bronchitis, which is characterized by the presence of productive coughing for at least three consecutive months during the last two consecutive years [4].

1.1.1 COPD: clinical features, risk factors, pathogenesis, treatment options

The clinical presentations in COPD patients are cough, sputum production, or dyspnea [5]. The airflow limitation of COPD is measured by spirometry, which is the most usable test of lung function for confirming a clinical diagnosis [4, 5]. According to GOLD Guidelines, the presence of a post-bronchodilator Forced Expiratory Volume in 1 second (FEV_1) per Forced Vital Capacity (FVC) ratio of less than 0.7 defines the presence of persistent airflow limitation for COPD [3-5]. In older adults, it is normally between 0.65 and 0.7 [6]. The GOLD criteria use the FEV_1 value (% predicted) to classify the severity of COPD into four stages: stage I mild (FEV_1 ≥80% of predicted), stage II moderate (FEV_1 50–80% of predicted), stage III severe (FEV_1 30–50% of predicted) and stage IV very severe (FEV_1 <30% of predicted) [3-6].

The risk factors for the development of COPD result from a combination of genetic susceptibility and exposure to environmental stimuli [3]. Smoking is the main risk factor, and around 90% of COPD patients are smokers [7]. However, one recent publication revealed that nearly 30% of all COPD patients have never smoked [8]. Other risk factors include childhood asthma and respiratory infections, exposure to indoor and outdoor air pollution, exposure to second-hand smoke and occupational exposure to dust and fumes [3, 5, 7, 8]. In addition, genetic factors are associated with the development of COPD, particularly a deficiency of alpha-1 antitrypsin (α1AT), which accounts for approximately 1- 2% of COPD cases [3, 7].

The main feature of COPD is irreversible airflow limitation. Pathologically, however, COPD is characterized by two main features: a narrowing and remodeling of small airways and the destruction of the alveoli, which both result from chronic inflammation [3, 9, 10]. Repeated exposure to cigarette smoke leads to the recruitment of neutrophils, macrophages and lymphocytes, which in turn release inflammatory mediators, such as cytokines, chemokines and proteases. The increased production of lung proteases, such as neutrophil elastase (NE), and the apoptosis of alveolar septal cells lead to the destruction of alveolar walls causing emphysema. Moreover, proteases, such as NE, cathepsin G and proteinase-3 promote mucus secretion by increasing the number of goblet cells, stimulating degranulation in these cells, in addition causing the enlargement of submucosal glands. Further, mucus hypersecretion, inflammation in the airway walls and lumen, and fibrosis formation around the small airways lead to the narrowing of these airways [11-13]. Clinical symptoms can develop in patients many years after starting smoking, with COPD commonly diagnosed among patients over the age of 50 years with the highest frequency at approximately 70 years [14].

Upon diagnosis, effective strategies to reduce symptoms, risks and limit the severity of exacerbations should be provided according to individualized evaluations. As COPD is characterized by airflow limitation, the first line treatments are short-acting and long-acting

bronchodilators, such as long-acting β-agonists (LABA) [15]. β2-agonists bind to β2-adrenoceptors on the cell membrane and activate a Gs protein [16], which activates intracellular adenylcyclase. Then, adenylcyclase converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), which activates protein kinase (PK). This leads to myosin dephosphorization causing muscle relaxation [17]. A second line treatment for COPD patients is the prescription of inhaled corticosteroids (ICS) to reduce inflammation [15]. When symptoms persist or worsen, ICS are combined with LABA. ICS are designed to cross the plasma membrane and activate glucocorticoid receptors (GR) in the cytoplasm, which either decreases pro-inflammatory gene expression or activates the transcription of anti-inflammatory genes. However, corticosteroids have a limited effectiveness in COPD [18]. It is also recommended that the patient stops smoking, reduces exposure to occupational and environmental risk factors, and gets an influenza vaccine annually [3]. There is still no curative therapy for COPD. Therefore, more research is needed to understand the effect of smoking on COPD and its pathogenesis, which can lead to the development of new targeted therapies for COPD.

1.2 Inflammation in COPD: characteristics and features.

Inflammation in COPD involves both innate and adaptive immune responses. This inflammation worsens during acute exacerbations and/or during bacterial or viral infection [19]. Exacerbations of COPD are strongly correlated with increase in hospitalization and mortality and a decrease in functional status [14]. Exposure to cigarette smoke or other irritants activates an innate inflammatory immune response, which delivers the primary protection against foreign particles. In the lower respiratory tract, this response is important for immune homeostasis [10, 19]. The innate immune system protects the respiratory tract via tight junctions between epithelial cells, which keep foreign particles on the surface. It also includes

the mucociliary clearance apparatus which clear these particles from the lower respiratory tract. Moreover, macrophages phagocytose deposited foreign particles on the bronchial and alveolar surfaces. These macrophages are carried in a thin liquid layer from the alveolar surface to the mucociliary clearance apparatus in the conducting airways, with this layer being secreted by type II pneumocytes. Chronic stimulation of the innate immune system by repeated exposure to cigarette smoke leads to a damage of the tight junctions, an increase in the production of mucus and a decrease in its clearance. There is also a recruitment of neutrophils, macrophages and dendritic cells to the lung. In addition, chronic exposure to cigarette smoke activates the adaptive immune system, which is the second line of defense primarily mediated by T and B lymphocytes [3, 19]. The inflammation in COPD patients, which persists even after smoking cessation, is largely due to memory T cells, bacterial colonization or autoimmunity [19].

1.2.1 Cigarette smoke and induction of inflammation

The main cause of COPD is cigarette smoke [3]. Cigarette smoke is a complex combination of thousands of chemicals (approximately 5685 individual components) of which about 158 have toxicological properties [20, 21]. The components with the highest correlations to carcinogenesis are polycyclic aromatic hydrocarbons (PAHs) and N-nitrosamine, while other components are known to cause pulmonary toxicity, such as free radicals, catecholes and aldehydes [22]. In the lungs, exposure to cigarette smoke induces inflammation in several cell types, including epithelial cells, fibroblasts and macrophages [23-25]. Cigarette smoke recruits inflammatory cells to the site of exposure, maintains their persistence, and increases inflammatory mediators, the net effect of which is damage to the airways and lungs [10, 19, 22].

1.2.2 Inflammatory cells and mediators

The cells that contribute to the inflammation in the lungs of COPD patients include both inflammatory cells, such as macrophages, neutrophils, dendritic cells and lymphocytes, and lung structural cells, which include epithelial cells, fibroblasts and endothelial cells. These cells secrete many inflammatory mediators that have been implicated in COPD [14, 19]. All of these cells work together to regulate inflammation both in COPD and in response to cigarette smoke.

1.2.2.1 Inflammatory cells

Macrophages:

Macrophages play a critical role in inflammation in COPD. The number of macrophages is increased in the airways, bronchoalveolar lavage (BAL) fluid, sputum, and lung parenchyma of COPD patients [26]. Cigarette smoke activates macrophages to produce inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukin-1β (IL1-β), IL-8, monocyte chemoattractant protein 1 (CCL2) and leukotriene (LT) B_4. These macrophages also secrete proteases, including matrix metalloproteinases (MMPs) 2, 9 and 12, NE, and cathepsins K, L and S [19]. In COPD patients, alveolar macrophages produce more inflammatory proteins and proteases at baseline compared to macrophages from non-COPD smokers, which is further elevated in COPD patients who smoke [19]. Alveolar macrophages in COPD show a deficiency in the phagocytosis of bacteria, which may be a factor in chronic bacterial colonization in the lower airways. These macrophages also show a reduction in the efferocytosis of apoptotic cells (*i.e.*, the taking up apoptotic cells), a feature that might contribute to the worsening of inflammation in COPD [19, 26].

Neutrophils:

Activated neutrophils are increased in the sputum and BAL fluid of COPD patients, and this is associated with the severity of the disease. However, few neutrophils are seen in the airways and lung parenchyma [19]. Cigarette smoke increases the number and activation of neutrophils in the respiratory tract. The recruitment of neutrophils to the airways and parenchyma, upon exposure to smoke, includes their initial adhesion to endothelial cells via E-selectin [14, 19]. In COPD, E-selectin is upregulated on endothelial cells in the airways. Then, neutrophils migrate into the respiratory tract in response to various neutrophil chemotactic factors, including LTB_4 and IL-8. These chemotactic mediators are increased in the airways of COPD patients, and are secreted from epithelial cells, alveolar macrophages, T cells, and from the neutrophils themselves. Activated neutrophils are a potent source of proteases, such as NE, cathepsin G, proteinase-3, MMP-8 and MMP-9, all of which contribute to the destruction of alveolar walls. NE, cathepsin G and proteinase-3 also promote mucus secretion from submucosal glands and goblet cells [14, 19].

Dendritic cells:

Activated pulmonary dendritic cells are increased in COPD patients, and this is also associated with disease severity immunity [19]. Cigarette smoke also increases dendritic cell survival. These cells are one of the main links between innate and adaptive immunity [19]. Dendritic cells are located within the epithelium and lamina propria [10] where they activate macrophages, neutrophils, and T and B lymphocytes [19]. Dendritic cells also pick up antigens, which are released from damaged tissue, or foreign antigens and process them before presenting them to lymphocytes in the draining lymph nodes [3, 10].

Lymphocytes:

T lymphocytes are increased in the lung parenchyma and peripheral and central airways of COPD patients [19, 27]. A greater increase in $CD8^+$ cells over $CD4^+$ cells has been observed [27]. The number of T-cells is correlated with the amount of alveolar destruction and the severity of airflow obstruction. $CD8^+$ cells trigger apoptosis in alveolar epithelial cells through the release of TNF-a, granzyme B and perforins [19]. T-cells were increased in a mouse model of cigarette- induced emphysema, and this was directly related to emphysema severity [11]. In COPD patients, B lymphocytes are also increased, especially in severe stages of the disease [19]. As COPD progresses, tertiary lymphoid aggregates develop around the small airways. These aggregates, called lymphoid follicles, include T and B cells [3, 10]. The number of lymphoid follicles are increased in COPD patients, particularly those with $FEV_1 < 50$ %, and this is one of the most prominent histological features of COPD. In these patients, lymphoid follicles surround about a third of the small airways [28].

1.2.2.2 Lung structural cells

Epithelial cells:

In COPD, airway and alveolar epithelial cells are important producers of inflammatory mediators and proteases. Cigarette smoke causes direct damage to these cells, which leads to the release of inflammatory mediators such as TNF-α, IL-1β and IL-8, as well as transforming growth factor β (TGF-β), the latter of which plays a role in fibrosis formation [11, 19]. Vascular endothelial growth factor (VEGF) has been shown to help alveolar cells withstand such damage; indeed, blocking of its receptors in rats stimulates the apoptosis of alveolar cells and to produce emphysema-like pathology [29]. In COPD, the level of VEGF is decreased, which may be a contributing factor to the development of emphysema [19]. The airway epithelium is also important in defending the airways from damage via the secretion of antioxidants and antiproteases such as secretory leukoprotease inhibitor (SLPI) that contributes to host defense

and tissue repair [11]. In emphysema, damage to the alveolar epithelium can decrease the level of SLPI production [30]. Exposure to cigarette smoke and noxious particles impairs the defense response mechanisms of the airway epithelium and increasing the sensitivity to infection [11, 19].

Endothelial cells:

Resting endothelial cells inhibit the coagulation of blood, regulate blood flow, control vessel-wall permeability and inhibit the recruitment of leukocytes. However, during inflammation, these cells fail to perform these functions [31]. In COPD patients, endothelial dysfunction is increased, and the expression of cell adhesion molecules is activated. The apoptosis of these cells is elevated, which may contribute to the development of emphysema, and pulmonary microvascular blood flow is reduced [32].

Fibroblasts:

Lung fibroblasts provide structure and support to the lungs by synthesizing and maintaining an extracellular matrix (ECM) [33]. Further, alveolar fibroblasts directly connect type II pneumocytes to endothelial cells, which provides a bridge for leukocyte migration [34, 35]. In chronic inflammation, the activation of fibroblasts leads to the production of many cytokines, chemokines and proteases. The activation of the nuclear factor (NF)-κB family of transcription factors in fibroblasts leads to an increase in the synthesis of IL-8, cyclooxygenase-2 (COX-2) and IL-6 [36]. In COPD, lung fibroblasts expressed a high level of intercellular adhesion molecule-1 (ICAM-1), which is involved in the recruitment of inflammatory cells [37]. In addition, the ability of lung fibroblasts from COPD in tissue repair response is reduced comparing to Normal fibroblasts [38]. Cigarette smoke itself activates lung fibroblasts, thereby increasing the production of COX-2 and IL-8 [24, 25].

1.2.2.3 Inflammatory mediators

In COPD, several inflammatory mediators have been implicated, including free radicals, cytokines, chemokines, and growth factors, which are derived from structural and inflammatory cells in the lung [19]. Here we will focus on two important mediators: COX-2 and IL-8.

COX-2:

COX-2 is an inducible enzyme that metabolizes arachidonic acid, which is released from the plasma membrane via phospholipase, and synthesizes prostaglandins, including prostaglandin (PG) D_2, $PGF_{2\alpha}$, PGE_2 and PGI_2, and thromboxane A2 [39]. PGE_2 production depends on the conversion of PGH_2 via microsomal prostaglandin E synthase-1 (mPGES-1) [24]. PGD_2, PGE_2 and PGI_2 play a main role in gastric cytoprotection, vasodilatation, renal homeostasis maintenance and platelet aggregation [39]. PGE_2 biological functions are mediated by the four different E prostanoid receptors (EP), EP_{1-4}, activation [40]. A study has shown that PGE_2-induced relaxation of human airway smooth muscle is mediated via activation of the EP_4 receptor [41], while PGE_2-induced stimulation of sensory nerves is mediated via activation of the EP_3 receptor [42]. PGE_2 has also an anti-inflammatory effect via activation of the EP_4 receptor. In this study, the absence of the EP_4 receptor in mice enhances airway inflammation in response to CS and lipopolysaccharide (LPS) [43]. On the other hand, PGE_2 has a role in inflammation, which participates in processes leading to redness, swelling and pain, which are the classic signs of inflammation. It increases blood flow into the inflamed tissue through increased microvascular permeability and vasodilatation causing redness and edema. PGE_2 also acts on peripheral sensory neurons and on central sites in the brain and spinal cord causing pain [44]. Moreover, in human lung fibroblasts exposed to cigarette smoke, COX-

2, mPGES and PGE_2 levels are increased [24], as well as lung fibroblasts from COPD patients, COX-2 and PGE_2 expression are increased [38]. In addition, a study showed that mPGES-1 level was increased in Smoker and COPD lung fibroblasts [45].

IL-8:

IL-8 is a chemokine that attracts immune cells to the site of the injury, such as neutrophils, monocytes and T-cell [46, 47]. High levels of IL-8 in the serum correlate with the increase in neutrophils in the circulation. IL-8 affects neutrophils including changing shape and exocytosis of stored proteins to cause the release of superoxide anions and hydrogen peroxide [46]. In response to stimuli such as LPS and IL-1, IL-8 is secreted into the extracellular space [48]. Cigarette smoke also increases IL-8 production from lung fibroblasts [25]. In COPD, IL-8 is increased in the plasma, sputum, airway epithelial cells and fibroblasts [49-51].

1.2.3 Molecular Regulation of Inflammatory Mediators

In response to smoke, COX-2 and IL-8 expression are regulated by the NF-κB family of transcription factors [52]. In addition, cigarette smoke phosphorylates p38 Mitogen-activated protein kinase (MAPK) and MAPK-activated kinase 2 (MK2), a downstream substrate of the p38 MAPK, which in turn increase IL-8 production [47]. COX-2 expression is also regulated by p38 MAPK upon cigarette smoke exposure [53]. Further, studies suggest using p38 MAPK inhibitors as a treatment for COPD. Nevertheless, potential problems may be arise from using p38 MAPK inhibitors [54]. Consequently, it is requisite to understand the molecular mechanisms regulating inflammation in response to cigarette smoke.

1.3 RNA binding proteins

The post-transcriptional regulation of messenger RNA (mRNA) is an important part of gene expression [55]. The mRNAs bind with RNA-binding proteins (RBPs) in cells to create messenger ribonucleoprotein (mRNPs) complexes. RBPs mediate the fate of mRNA, including the splicing and maturation of precursor mRNA (pre-mRNA). They also mediate the exporting, localization, stabilization and translation of mRNA into protein, and thus provide a link between these processes [55, 56]. The coordination of post-transcriptional processes necessitates the appropriate functioning of these RBPs. When they do not function appropriately, disease may arise [55]. For example, CUG triplet repeat RNA-binding protein 2 (CUGBP2), which is RNA binding protein of the CEL family, stabilizes *Cox-2* mRNA, but does not induce its translation [57]. However, some RBPs destabilizes mRNA such as AU-binding factor 1 (AUF1), also known as heterogeneous nuclear ribonucleoprotein D (hnRNP D) [58]. One of the best known RBPs with a regulatory influence on mRNAs is human antigen R (HuR).

1.3.1 Hu/ELAV family: HuR

The ubiquitously-expressed HuR, also known as HuA or embryonic lethal abnormal vision-like protein 1 (ELAVL1), belongs to the embryonic lethal abnormal vision (ELAV) family of RBPs [59, 60]. Other members of this protein family include HuB, HuC and HuD, which are neural-specific proteins [60]. HuR contains three RNA recognition motifs (RRMs): RRM-1 and RRM-2 bind to adenylate-uridylate (AU)-rich elements, while RRM-3 binds to the poly (A) sequence of target mRNAs. RRM-3 is also involved in the stabilization of the HuR-RNA complex as well as protein-protein interactions [60, 61]. HuR interacts with T-cell Intracellular Antigen 1 (TIA-1), which is a RBP, and competitively regulate many target mRNAs [62]. The nuclear shuttling domain (HNS) of HuR is localized in the hinge region between RRM-2 and RRM-3, which is important for the shuttling of HuR out of and back into

the nucleus [59, 61]. Beside the HNS region of HuR, the mobilization of HuR is regulated by transportins 1 and 2, the chromosome region maintenance 1 (CRM1) and importin-1α, which are components of nuclear transport machinery [59]. HuR targets mRNAs have U- or AU-rich elements in the 3'-untranslated region (UTR). Thus, HuR binds to these elements and influences the stability and/or translation of target mRNAs, pre-mRNA splicing and nucleocytoplasmic translocation of the target mRNAs [60]. HuR is also mainly located in the nucleus in resting cells, which can be transported to the cytoplasm along with bound mRNA in response to stimuli, such as UVB and radiation [59-61]. The target mRNAs which HuR stabilizes encode proteins involved in the proliferation, differentiation, migration and apoptosis of cells. They also encode proteins involved in inflammation, fibrosis and cancer [59, 61, 63-66].

1.3.2 The control of HuR function

HuR abundance and its post-translational modifications affect HuR binding to target mRNAs, all effectively impacting HuR function [59, 60]. The abundance of *HuR* mRNA is regulated at the transcriptional level by NF-κB and smad [67, 68]. HuR also stabilizes its own mRNA [69]. However, microRNA (miRNA) miR-519 and miR-125a inhibit HuR translation [70, 71]. Further, the levels of HuR protein have been observed to decrease transiently in response to heat shock by ubiquitination of HuR at residue Lys-182 followed by proteolysis [59, 60]. Finally, HuR can be cleaved by Fas associated via death domain (FADD), caspase-8 and caspase-3 in response to lethal damage, such as that caused by staurosporine. The products of this cleavage are then involved in apoptosis [72]; the larger product of HuR cleavage promotes myogenesis in muscle cells [73].

The post-translational modifications of HuR are phosphorylation or methylation of HuR at different residues, which regulate its localization and/or binding to target mRNAs [59,

60]. For example, in cells exposed to γ-irradiation, p38 phosphorylates HuR, induces HuR translocation to the cytoplasm and subsequent binding to *p21* mRNA, which is cyclin-dependent kinase inhibitor that cause G_1/S cell cycle arrest [74]. In macrophages exposed to LPS, coactivator-associated arginine methyltransferase 1 (CARM1) methylates HuR, which in turn stabilizes *TNF-α* mRNA [75]. The post-translational modification of HuR within RRMs affects its binding to target mRNAs, whereas a modification around the hinge region affects its subcellular localization [59, 60].

1.3.3 HuR: roles in inflammation

In inflammation, HuR binds to mRNA encoding inflammatory proteins, thereby promoting their translation. For example, HuR stabilizes *Cox-2* mRNA in fibroblasts [76] and induces COX-2 expression in many types of cancer, including lung cancer [77, 78]. Moreover, down-regulated HuR decreases COX-2 levels in response to serum deprivation [79]. In addition, in response to IL-1β, HuR stabilizes *Il-8* mRNA and induces its translation [80]. Because HuR stabilizes mRNAs encoding inflammatory proteins, it is involved in the pathogenesis of inflammatory diseases, such as inflammatory bowel disease [59]. However, the expression and localization of HuR in COPD or its molecular regulation by cigarette smoke is not known.

CHAPTER 2: HYPOTHESIS AND AIMS

HYPOTHESIS

Increased expression and cytoplasmic translocation of HuR in COPD derives inflammatory protein production.

AIMS:

AIM 1. Assess HuR expression and localization in the human lung tissue and cells of Normal, Smoker and COPD subjects.

AIM 2. Determine the effect of HuR on COX-2 and IL-8 proteins production in Normal lung fibroblasts exposed to cigarette smoke extract (CSE).

CHAPTER 3: EXPERIMENTAL PROCEDURES

3.1 Chemicals

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Actinomycin D (ActD) was obtained from Enzo Life Sciences.

3.2 Subjects and Specimens

Lung tissue used in this study was obtained from subjects undergoing lung resection surgery at McMaster University. This study was approved by the Research Ethics Board of St, Joseph's Healthcare Hamilton and informed written consent was obtained from each patient. The study population included those who current smokers with COPD (COPD), Smokers without COPD (Smoker), or non-smokers without COPD (Normal). The clinical characteristics of the subjects which the lung tissues and fibroblasts were derived are given in Table 1 [81]. None of the patients from which the lung tissue was derived received chemotherapy or radiotherapy before surgery.

Table 1. Subject characteristics

	Normal	**Smoker**	**COPD**
No. of subjects	6	4	9
Age	67.2±6.9	55.7±1.1	68.3±4.5
Gender (M/F)	3/3	1/3	5/4
Pack years of smoking	0.0	32.5±4.8	46.2±8.9
FEV_1 (%)	99.6±8.5	89.3±8.4	73.6±5.9
FVC (%)	97.4±8.5	94.3±6.7	91.13±7.5
FEV_1/FVC (%)	80.7±4.1	75.4±2.5	61.04±4.2

3.3 Cell Culture

Primary lung fibroblasts (HLF) were isolated from cancer-free lung tissue by explant procedure [82]. Once we received the lung tissue, aseptic techniques were followed to minimize contamination of the primary culture. Small pieces of lung tissue were placed on the bottom of sterile plastic tissue culture dish. Five pieces were covered by glass cover slip and secured by sterile grease. Then, tissues were covered by RPMI20 (RPMI ‘’Richter’s improved MEM insulin’’; Life Technologies, Gaithersburg, MD) supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and incubated in humidified 5% CO_2/95% air at 37˚C. The plates were checked every day and the media was changed three times a week for a period of approximately four weeks. Under these conditions, most non-fibroblast cells, including epithelial and endothelial cells, die whereas the fibroblasts grow and proliferate throughout the first two weeks of culture. When the fibroblasts were outgrown, the cells were passaged for further experiments. The characterization of the fibroblasts was based on morphology and vimentin expression [81, 82]. Subsequent to the characterization, the cells were expanded and either were frozen and stored in liquid nitrogen or maintained and sub-cultured in 10% MEM for experiments as outlined below. All fibroblast strains were cultured and analyzed at the same time and were within one passage (3-4) to assess the basal expression levels of HuR. Experiments were conducted with fibroblasts from two different individuals of each patient group and within the passage five to eleven. BEAS-2B cell line, derived from human airways epithelial cell (ATCC, Manassas, VA), was maintained in 10% DMEM. AM-MHS cell line, derived from alveolar macrophage (ATCC, Manassas, VA), was maintained in 10% RPMI.

3.4 Multiplex Immunohistochemistry (mIHC)

Immunohistochemistry was performed in lung tissues from Normal, Smoker and COPD subjects (n=3-4/group) by Discovery Ultra Ventana (Roche, CA). Formalin-fixed paraffin-embedded blocks were cut to three consecutive 4-μm-thick sections. One section was used for routine Hematoxylin and Eosin stain, the second section for mIHC and the third for negative control staining. All sections were subjected to the fully automated multiplexing IHC assay developed by Ventana (Roche, CA). Briefly, the slides were deparaffinized at 69˚C and pretreated with CC1 (EDTA) for 24 minutes at 95˚C. Then, Discovery Inhibitor was added for 4 minutes. The slides were incubated with primary antibody for HuR (1:100; Santa Cruz, CA) for 15 minutes at 37˚C. The secondary antibody coupled to horseradish peroxidase (HRP) (OMap anti-mouse HRP, Roche, CA) was incubated for 16 minutes. Immunodetection was performed with 3,3'-diaminobenzidine (DAB, Roche, CA). Then, the primary antibody for vimentin (1:50; Cell Signaling Technologies, CA) was incubated for 16 minutes at 37˚C followed by the secondary antibody UltraMap (anti- rabbit alkaline phosphatase) and incubated for 16 minutes at 37˚C. Immunodetection was performed with DISCOVERY Yellow kit. Next, the slides were incubated with the triple stain using the primary antibody for cytokeratin 19 (Roche, CA) for 16 minutes at 37˚C. The secondary antibody was multimer HRP (OMap anti-mouse HRP, Roche, CA) and was incubated for 16 minutes. Immunodetection was performed with DISCOVERY Purple detection. Finally, nuclei were subsequently visualized with hematoxylin. The images were taken with Aperio ImageScope.

3.5 Preparation of Cigarette Smoke Extract (CSE)

Research grade cigarettes (3R4F) with a filter were acquired from the Kentucky Tobacco Research Council (Lexington, KT). Each cigarette contains 0.73 mg of nicotine, 9.4 mg of tar, and 12.0 mg of CO as described by the manufacturer. CSE was produced as previously described [76, 83]. Briefly, CSE was prepared by bubbling smoke from a cigarette

through 15 ml of serum-free MEM, sterile-filtering with a 0.45-μm filter (25-mm Acrodisc; Pall Corp., Ann Arbor, MI) and was used within 30 minutes of preparation. An optical density of 0.65 (320 nm) was regarded as 100% CSE [76, 83] which was diluted in serum-free MEM to the appropriate concentration.

3.6 Western Blot

Fibroblasts were grown to approximately 70-80% confluence and cultured with serum-free MEM for 18 hours before the treatment. Total cellular protein was extracted using RIPA lysis buffer (Thermo Scientific, Rockford) and Protease Inhibitor Cocktail (PIC, Roche, US); nuclear and cytoplasmic fractions were extracted by using a nuclear extract kit (Active Motif, Carlsbad, CA). Five to ten μg of protein lysate were subjected to 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels and transferred onto Immuno-blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA) as previously described [84]. Then, the membrane was blocked for one hour at room temperature in blocking solution (5% w/v of nonfat dry milk in 1x PBS/0.1% Tween-20). Antibodies against HuR (1:2000; Santa Cruz, CA), COX 2 (1:1000; Cell Signalling Technologies, CA), Lamin A/C (1:1000; Cell Signalling Technologies, CA) and Tubulin (1:50000; Sigma, CA) were used. The secondary antibodies goat anti-rabbit IgG, HRP-linked (1:10000, Cell Signaling Technologies, CA) and HRP-conjugated horse anti-mouse IgG (1:10000, Cell Signaling Technologies, CA) were used. Detection of the change in protein levels was catalyzed by ClarityTM western ECL substrate (Bio-Rad Laboratories, Mississauga, ON) or AmershamTM western ECL substrate (GE Healthcare, Italy). Protein bands were visualized using a ChemiDoc™ MP Imaging System (Bio-Rad, CA). Densitometric analysis was performed using Image Lab™ Software Version 5 (Bio-Rad, CA). Protein expression was normalized to Tubulin and the data presented as the fold-change relative to the untreated condition.

3.7 HuR-siRNA knock-down in human lung fibroblasts

Normal human lung fibroblasts were seeded at $10x10^4$ cells/cm^2 and transfected with 60 nM of siRNA against HuR (Santa Cruz, CA) or non-targeting control siRNA (Santa Cruz, CA) in accordance with manufacturer's instructions. Six hours after the transfection, 20% MEM medium was added on the cells. In 24 hours, the cells were treated with serum-free MEM medium for 18h, followed by exposure to 2% CSE. Confirmation of HuR knock-down was done by western blot within 50 and 66 hours after transfection.

3.8 Enzyme-linked immunosorbent assay (ELISA)

The concentration of interleukin-8 (IL-8) in the cell culture supernatant was determined by ELISA (Human IL-8 ELISA MAX™ Standard Set, BioLegend, U.S) according to the manufacturer's instructions. The absorbance was read at 450 nm and 570 nm within fifteen minutes by iMark microplate reader (Bio-Rad Laboratories, Mississauga, ON).

3.9 Quantitative RT-PCR (qRT-PCR)

Total RNA (mRNA) was prepared from Normal HLFs by using Trizol according to the manufacturer's instructions. Quantification was conducted on a Nanodrop 1000 spectrophotometer (infinite M200 pro, TECAN, CA). Reverse transcription of RNA was carried out using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Mississauga, ON). Then, the mRNA levels of *HuR, Cox-2, Il-8* and *S9* were analyzed using this cDNA template and gene-specific primers (Table 2). Quantitative PCR (qPCR) was performed by addition of 1 µl cDNA and 0.5 µM primers with SsoFast™ EvaGreen® (Bio-Rad Laboratories, Mississauga, ON), and PCR amplification was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad, CA). Thermal cycling was initiated at 95°C for 3

minutes and followed by 39 cycles denaturation at 95°C for 10 seconds and annealing at 59°C for 5 seconds. Gene expression was analyzed using the ΔΔCt method, and results are presented as fold-change normalized to housekeeping gene (*S9*).

Table 2. primer sequences used for qRT-PCR analysis.

Gene	Forward Primer Sequence	Reverse Primer Sequence
HuR	AAC GCC TCC TCC GGC TGG TGC	GCG GTA GCC GTT CAG GCT GGC
Cox-2	TCA CAG GCT TCC ATT GAC CAG	CCG AGG CTT TTC TAC CAG A
Il-8	GAT GTC AGT GCA TAA AGA CAT ACT CCA A	GCT CTC TTC CAT CAG AAA GCT TTA CAA TA
S9	CAG CTT CAT CTT GCC CTC A	CTG CTG ACG CTT GAT GAG AA

3.10 Determination of mRNA stability

SiCtrl and siHuR- transfected Normal HLF were prepared for treatment as described in section 3.7. Then, cells were exposed to 2% CSE for 24 hours followed by the treatment of ActD (1 μg/ml), an inhibitor of RNA synthesis [76, 85], for 30 minutes, 1 hours, 3 hours or 6 hours. Total RNA was harvested and qPCR was performed as described in section 3.9 to determine the remaining levels of *Cox-2* and *Il-8* mRNAs. ActD concentration used in this experiment did not affect cell viability [76].

3.11 Imaging flow cytometry

Normal and COPD HLFs were grown to approximately 70-80% confluence and cultured with serum-free MEM for 18 hours before the treatment. Cells were exposed to 2% CSE for 4 hours. Then, cells were trypsenised, collected and prepared according to the manufacturer's instructions. Briefly, after washing cells with 1x PBS-0.2% BSA, cells were fixed with 4% Paraformaldehyde (PFA) for 20 minutes at room temperature. After incubation,

1x PBS-0.2% BSA was added, spun down at 400xg at 4˚C for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 400 µl 1x PBS-0.2% BSA, spun down at 400xg at 4˚C for 5 minutes, and again the supernatant was discarded. Then, the pellet was resuspended in 100 µl of 1x Permeabilization Buffer (BD Perm/Wash™ Buffer, eBioscience™), and was incubated for 15 minutes at room temperature. After incubation, 1 µl of HuR-PE (1:100, BD Pharmingen™) was added and incubated for 30 minutes at 4˚C in the dark, and then 1 ml of 1x Permeabilization Buffer was added. The samples were centrifuged, and the pellet was resuspended in 75 µl of 1x PBS-0.2% BSA. The samples were filtered and kept in the dark. Before acquiring the samples in the flow cytometer, 5 µl Hoechst (1: 20,000, Hoechst 3342, Thermo Scientific, Rockford) was added. In each experiment 20,000 events for each sample were acquired using a 12 channel Amnis® brand ImageStream®^{X} Mark II (EMD Millipore) imaging flow cytometer equipped with the 405 nm and 488 nm lasers. Samples were gated to remove debris, and at 20,000 event/ sample were analyzed using IDEAS®. After gated the cytoplasmic fraction, the intensity feature was used to evaluate HuR expression.

3.12 Statistical Analysis

Using GraphPad Prism 6 (v. 6.02; La Jolla, CA), statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test to assess differences between Normal, Smoker and COPD subjects. Groups of two were analyzed by two-tailed unpaired t-test. A two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test was used to evaluate differences between treatment groups of more than two. Results are presented as mean ± standard error of the mean (SEM) of the fold-changes compared to control cells. In all cases, a p value < 0.05 is considered statistically significant.

CHAPTER 4: CONTRIBUTION OF CO-AUTHORS

This thesis is based on my original work and is presented as a manuscript currently in preparation for peer-review and submission.

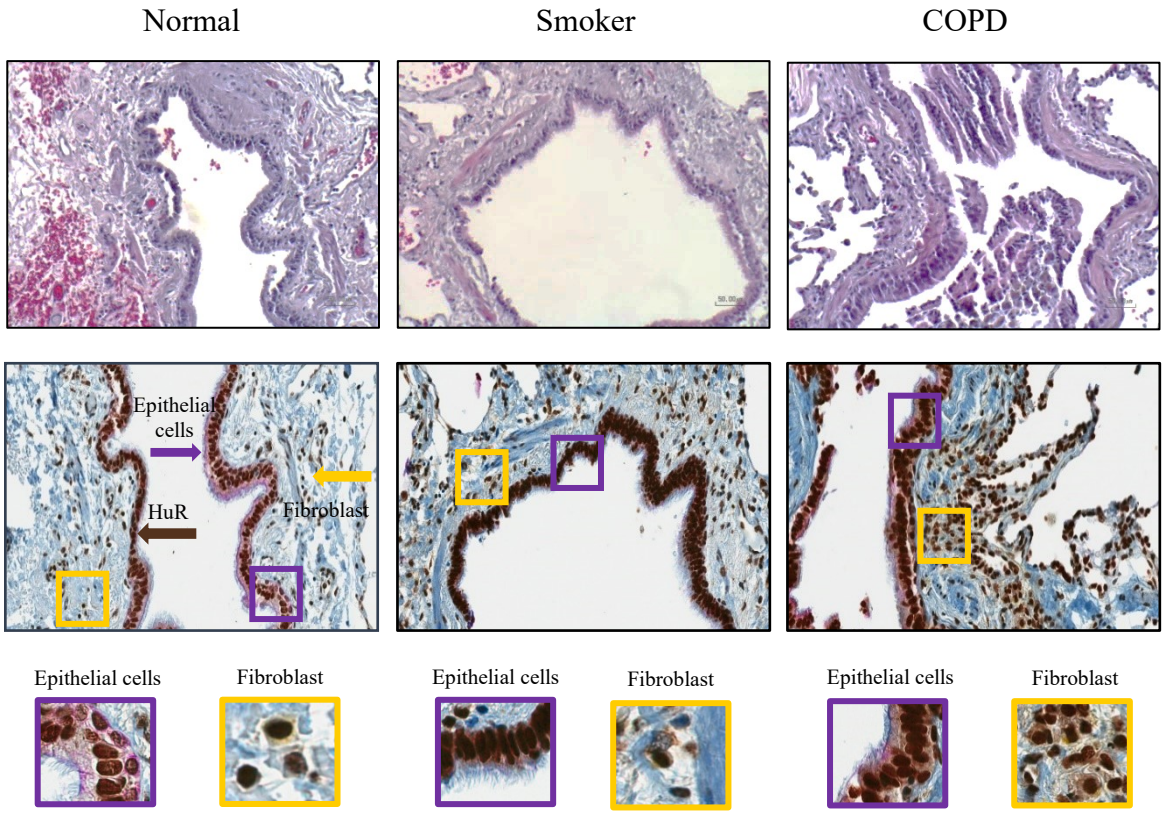
1- The human samples that were used in this project were provided from **Dr. Parameswaran Nair** in the Department of Medicine, McMaster University.
2- **Dr. David H. Eidelman:** provided assistance with experimental design and with manuscript editing.
3- **Dr. Qutayba Hamid:** provided assistance with manuscript editing.
4- **Dr. Carolyn Baglole:** provided intellectual support for planning and designing experiments, in addition to editing my thesis.

CHAPTER 5: RESULTS AND FIGURES

5.1 Cytoplasmic expression of HuR is increased in Smoker and COPD- derived lung tissue.

HuR has a pathogenetic role in inflammation and apoptosis [59] and is increased in lung cancer [77]. However, a role for HuR in COPD is not known. Therefore, we first asked whether HuR expression and cytoplasmic localization is increased in lung tissue derived from, COPD subjects. To answer this question, we used human lung tissue from Normal (N = 4), Smoker (N = 3) and COPD (N = 4) subjects. First, the lung structure was assessed by hematoxylin and eosin (H&E) staining to find optimal tissue quality to proceed with mIHC (Figure 1A. Top Panel). We then stained the next sequential slide using mIHC to detect HuR (brown), epithelial cells (purple) and fibroblasts (yellow) using antibodies against HuR, cytokeratin 19 and vimentin, respectively. We observed that in Normal tissue, HuR was localized primarily in the nucleus of epithelial cells and fibroblasts. Cytoplasmic HuR was noticeably weak in both cell types (Figure 1A. Bottom Panel and inset). Interestingly, HuR expression was dramatically increased in Smoker and COPD-derived lung tissue compared to Normal tissue (Figure 1A. Bottom Panel). Moreover, cytoplasmic localization of HuR in epithelial cells and fibroblasts was considerably higher in Smoker and COPD tissue compared to the Normal (Figure 1A. Bottom Panel). Finally, we found that HuR expression was higher in macrophages from Smoker and COPD subjects, including cytoplasmic HuR levels (Figure 1B). Altogether these data suggest that the expression and cytoplasmic translocation of HuR are increased in Smoker and COPD subjects comparing to Normal subjects.

A. Lung tissue



B. Macrophages- mIHC

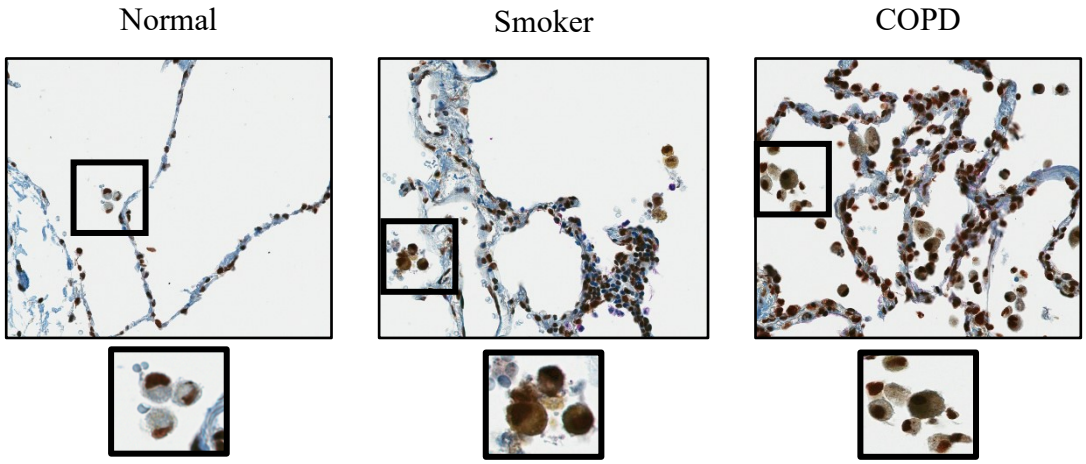


Figure 1. Cytoplasmic expression of HuR increased in lung tissue and macrophages from Smoker and COPD subjects. A. Lung tissue (Top Panel): Representative image (20x) of haematoxylin and eosin (H&E)-stained Normal, Smoker and COPD lung tissues. Bottom Panel: Lung tissue was stained with mIHC: HuR was stained with brown color, Cytokeratin 19 expression (a marker of epithelial cells), was stained with purple color and vimentin, (a marker for fibroblasts), was stained yellow. There was more HuR in the cytoplasm of epithelial cells and fibroblasts from Smoker and COPD subjects comparing to these cells from Normal individuals. **B.** Macrophages- mIHC: There was an increase in cytoplasmic HuR in macrophages from Smoker and COPD. The pictures were taken by Aperio ImageScope with 20x, and 40x for higher magnification. Images are representative for 3 to 4 subjects/ group.

5.2 Total expression and cellular localization of HuR is similar between Normal, Smoker and COPD-derived lung fibroblasts

Next, we derived primary lung fibroblasts (HLF) from Normal, Smoker and COPD subjects as previously described [82]. Our published data showed that HuR is expressed in Normal HLF [76]. Therefore, we examined the total expression of HuR in Normal, Smoker and COPD HLF by western blot (Figure 2A). Densitometric analysis of HuR protein indicated that there was no significant difference in total HuR expression between the three groups (Figure 2B). We then isolated the nuclear and cytoplasmic fraction, and evaluated the expression of HuR in the cytoplasm of HLF from the three groups by western blot. The purity of the cell fractions was assessed by using an antibody against Lamin A/C, as a marker of nuclear fraction, and an antibody against Tubulin, a cytosolic protein. Despite some contamination in the cytoplasmic fraction by Lamin A/C (Figure 2C), the analysis (HuR/Tubulin) showed an increase in HuR translocation to the cytoplasm by two-fold change in HLF derived from Smokers (Figure 2C and 2D). However, cytoplasmic HuR was not increased in COPD HLF relative to Normal HLF (Figure 2C and 2D). Together these data indicate that there is no significant difference in the total and the cytoplasmic expression of HuR between Normal, Smoker and COPD HLF.

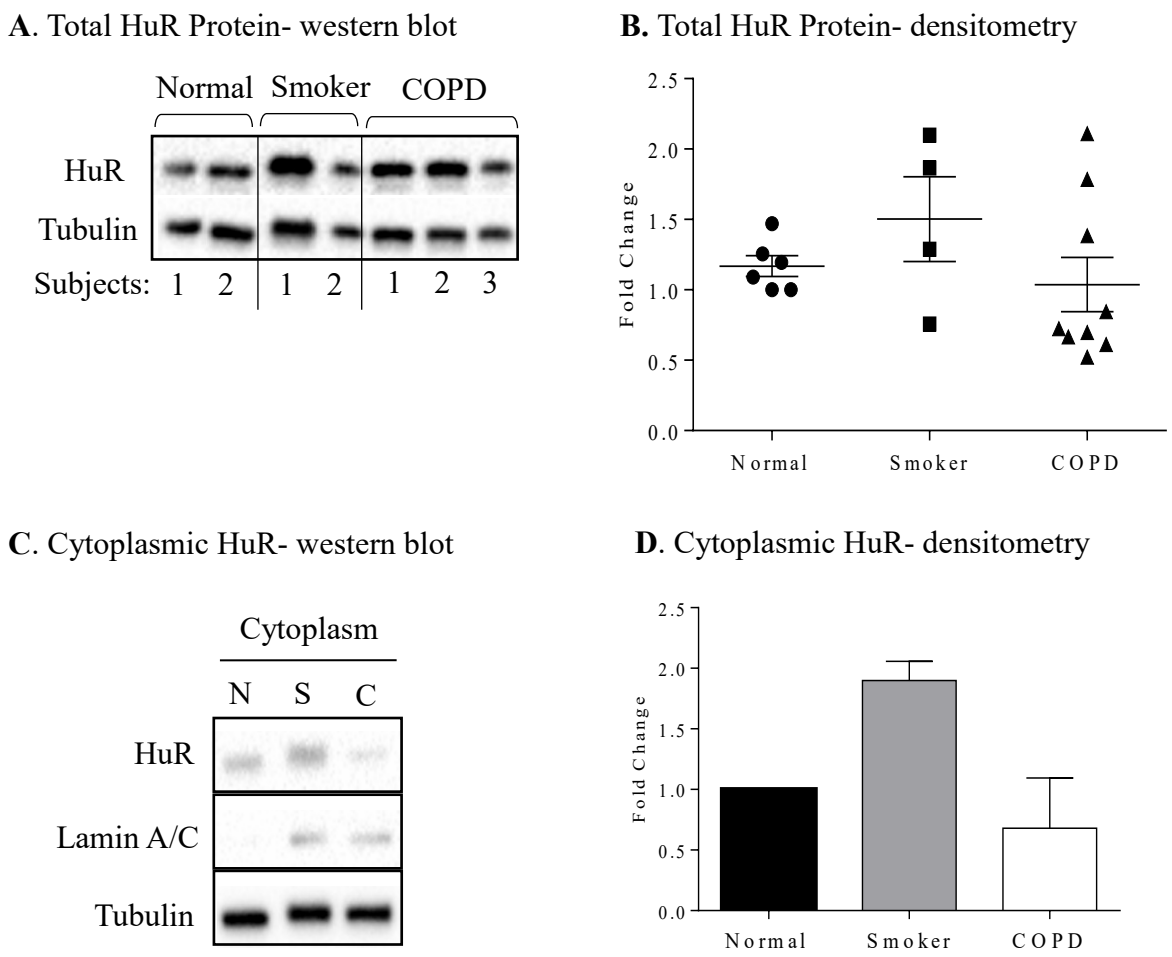
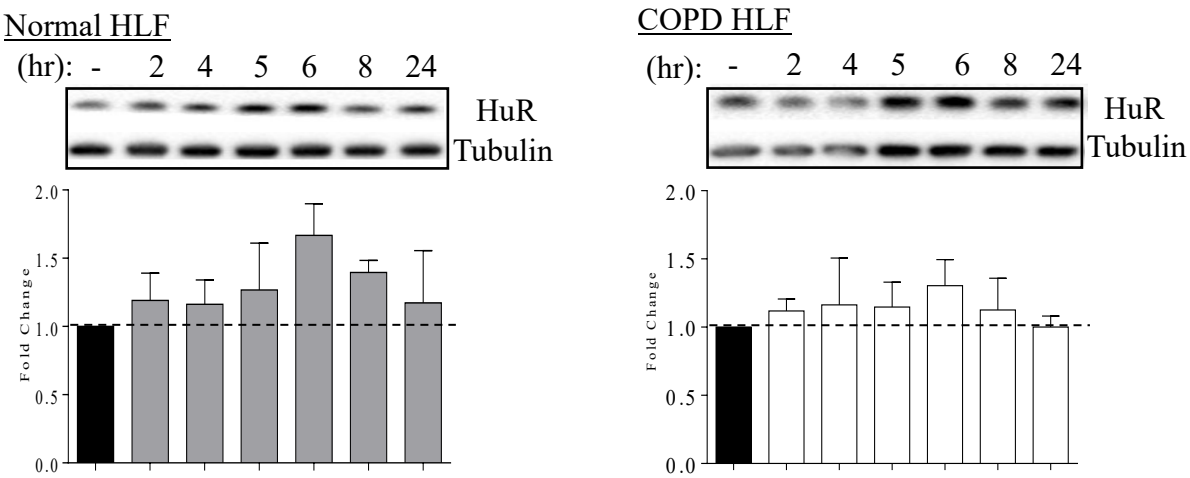


Figure 2. Total HuR expression and localization in Normal, Smoker and COPD HLF. A. Total HuR Protein- western blot: HLF from Normal, Smoker and COPD subjects were harvested for western blot. HuR protein expression was detected at the predicted MW of 34 kDa. The number of subjects used (Normal = 6; Smoker = 4; COPD = 9); representative western blot is shown. Tubulin was used as loading control. **B.** Total HuR Protein- densitometry: Densitometric analysis showed that there was no significant difference in total expression of HuR between Normal, Smoker and COPD HLF. **C.** Cytoplasmic HuR- western blot: The number of subjects used (Normal = 2; Smoker = 2; COPD = 2); a representative western blot is shown. Lamin A/C is a nuclear marker, while Tubulin is a cytoplasmic marker. **D.** Cytoplasmic HuR- densitometry: HuR expression in the cytoplasm is slightly- but not significantly-increased in Smoker HLF. Results are expressed as the mean ± SEM.

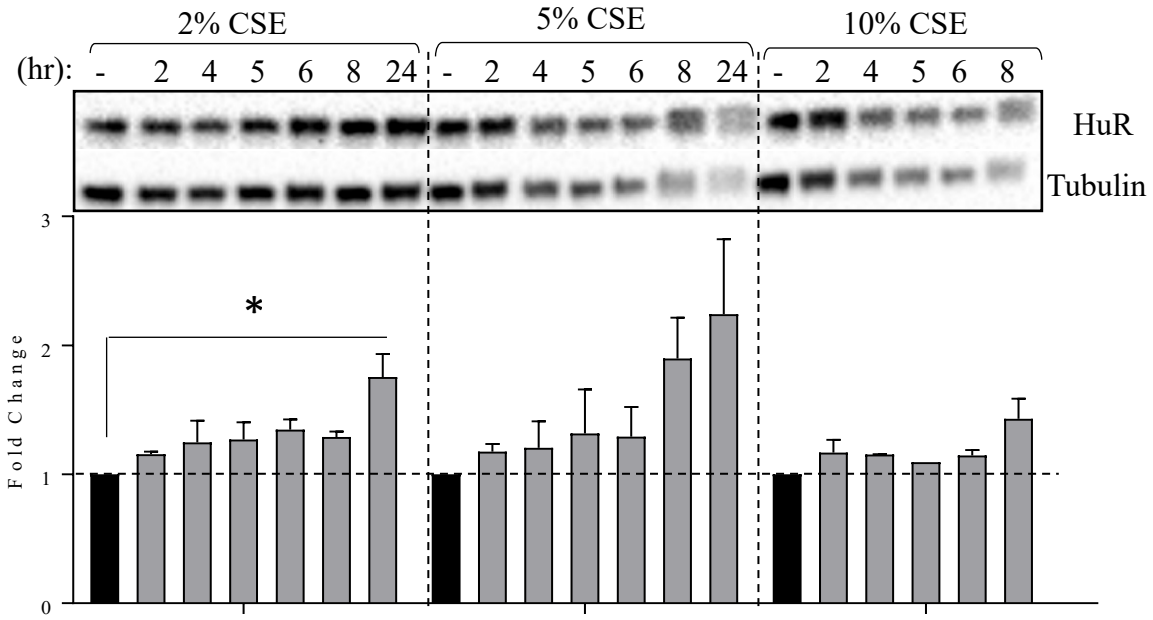
5.3 Total HuR expression in Normal and COPD-derived lung fibroblasts in response to cigarette smoke extract (CSE).

The effect of cigarette smoke (CS) on the expression of HuR is not known. Hence, we evaluated the effect of CS on the expression of HuR in HLF by treating cells with increasing concentrations of CSE (1%, 2%, 5% and 10%) for 2h, 4h, 5h, 6h, 8h and 24h. Protein levels of HuR did not increase significantly in Normal and COPD HLF treated with 1% CSE (Figure 3A). There was however a significant increase in HuR in response to 2% CSE in Normal HLF, but was not significant in COPD HLF at 24h after treatment (Figure 3B and Figure 3C). Interestingly, 5% and 10% CSE also did not yield a significant change in HuR levels (Figure 3B and 3C). *HuR* mRNA was also not changed in response to 2% CSE (Figure 4). These data indicate that CSE increases the expression of HuR protein in primary HLF. Because 2% CSE slightly increased the total expression of HuR in HLF from Normal and COPD subjects (without decreasing cell viability- data not shown) further experiments were conducted with 2% CSE.

A. Total HuR Protein -1% CSE



B. Total HuR Protein in Normal HLF



C. Total HuR Protein in COPD HLF

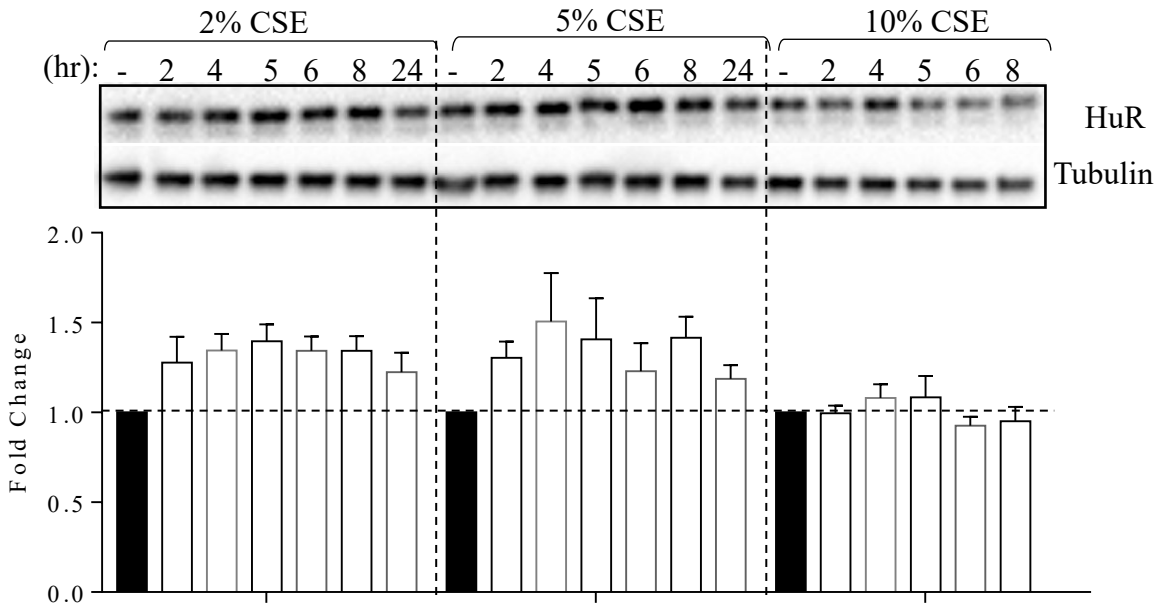


Figure 3. Total HuR expression in cigarette smoke extract (CSE)-exposed Normal and COPD HLF. A. Total HuR Protein- 1% CSE: Densitometric analysis showed that there was a slight but not statistically significant increase in total HuR expression in Normal and COPD HLF exposed to 1% CSE for 6h. Results are expressed as the mean ± SEM of 2-4 independent experiments (the number of subjects used: Normal = 2 and COPD = 2). **B.** Total HuR Protein in Normal HLF: Densitometric analysis indicated that there was an increase in total HuR protein expression in response to 2% CSE for 24h (*$p < 0.05$). HuR protein was increased slightly, but not statistically significant when cells exposed to 5% CSE for 4h, 5h and 6h, while HuR protein showed no difference when cells exposed to 10% CSE for 2h, 4h, 5h and 6h. Results are expressed as the mean ± SEM of 2 independent experiments (2 different Normal subjects). **C.** Total HuR Protein in COPD HLF: Densitometric analysis indicated that there was a slight increase in total HuR protein expression in response to 2% CSE. HuR protein was increased slightly, but not statistically significant, when cells exposed to 5% CSE for 4h, 5h and 6h, while HuR protein showed no difference when cells exposed to 10% CSE for 2h, 4h, 5h and 6h. Results are expressed as the mean ± SEM of 4 independent experiments (2 different COPD subjects).

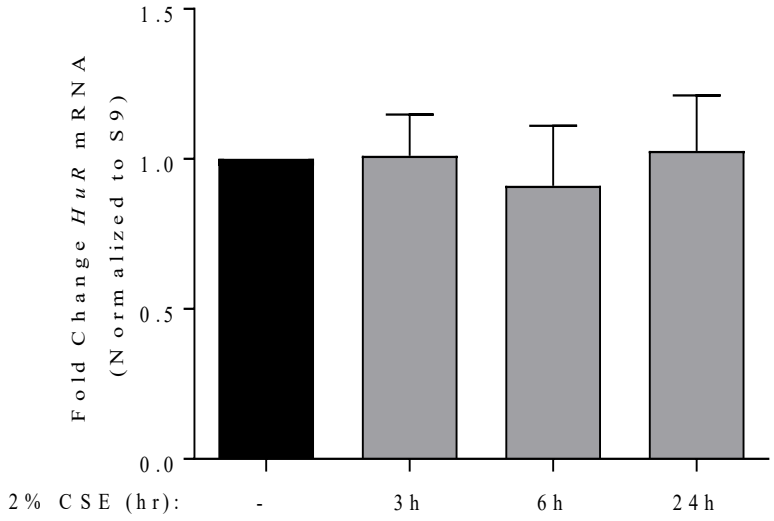
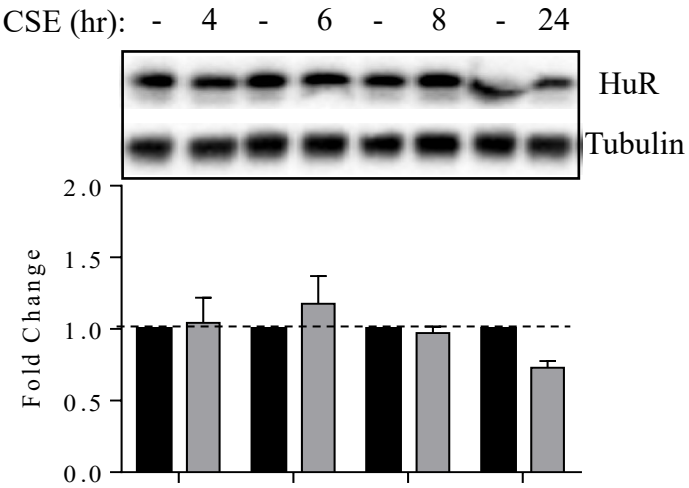


Figure 4. *HuR* mRNA do not change in response to 2% CSE. There was no significant difference in *HuR* mRNA in Normal HLF after exposure to 2% CSE for 3h, 6h and 24h. Results are expressed as the mean ± SEM of 4 independent experiments (HLF used from one Normal subject).

5.4 HuR protein expression does not change in BEAS-2B and AM-MHS in response to 2% CSE.

To assess the effect of CSE on HuR expression in other lung cells, we utilized the human airways epithelial cell line BEAS-2B and alveolar macrophage cell line AM-MHS. These cells were treated with 2% CSE for 4h, 6h, 8h, 24h and 48h. In both BEAS-2B cells and AM-HMS cells, densitometric analysis showed no difference in the total expression of HuR in response to 2% CSE (Figure 5A and 5B). Collectively, CSE does not increase the total expression of HuR in BEAS-2B and AM-MHS cell lines.

A. BEAS-2B



B. AM-MHS

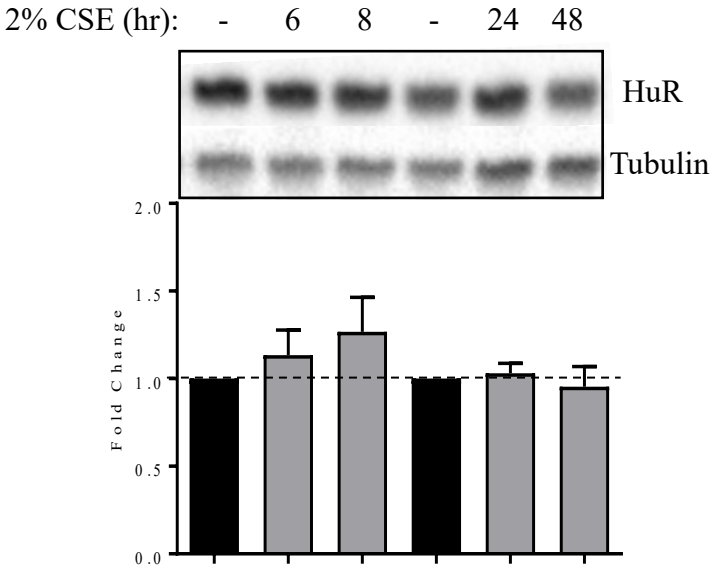


Figure 5. Cigarette smoke extract (CSE) does not increase the total expression of HuR in BEAS-2B and AM-MHS. A. BEAS-2B: There was no change in the total expression of HuR in BEAS-2B exposed to 2% CSE at different time points. Results are expressed as the mean ± SEM of 2 independent experiments. **B.** AM-MHS: there was no change in the total expression of HuR in AM-MHS in response to 2% CSE. Results are expressed as the mean ± SEM of 3 independent experiments.

5.5 Normal and COPD-derived lung fibroblasts exposed to 2% CSE for four hours increase the cytoplasmic expression of HuR.

Next, we asked whether CSE increases the cytoplasmic translocation of HuR in Normal and COPD HLF. First, we treated Normal and COPD HLF with 2% CSE for 4h, isolated the nuclear and cytoplasmic fraction, and detected HuR expression by western blot. We found an increase in HuR translocation to the cytoplasm by two-fold change in the 2% CSE-treated HLF comparing with the untreated. This increase was similar between the 2% CSE-treated Normal and COPD HLF (Figure 6A and 6B).

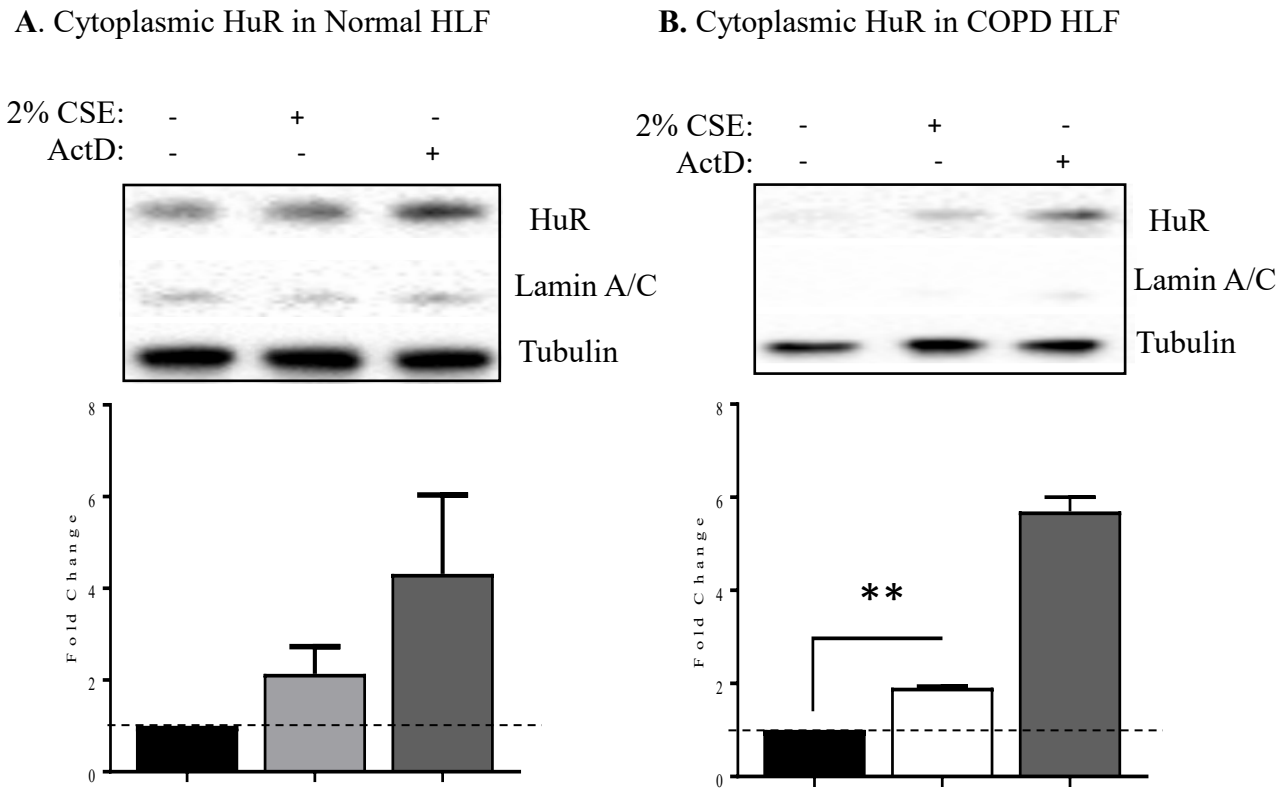
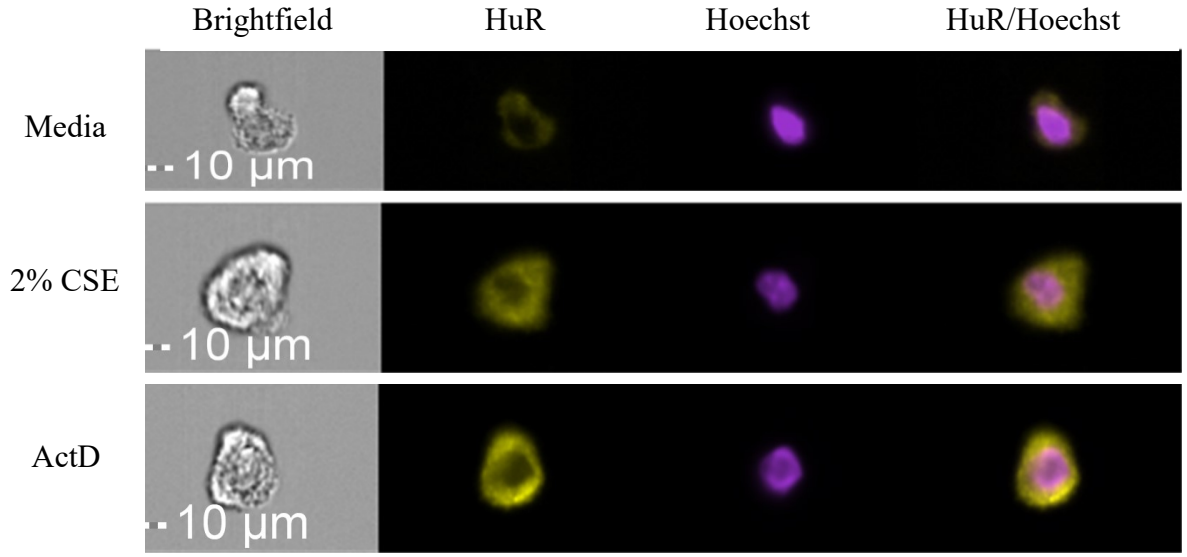


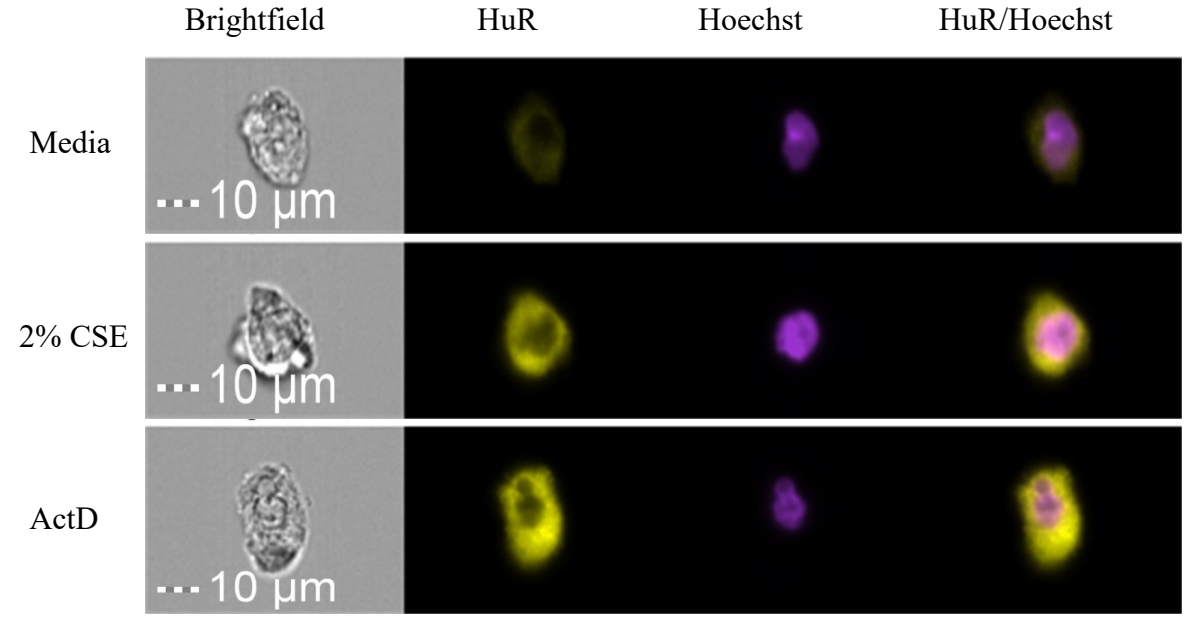
Figure 6. Cytoplasmic HuR protein levels increased in 2% CSE-exposed Normal and COPD HLF for four hours. A. Cytoplasmic HuR in Normal HLF: There was an increase, but not statistically significant, in HuR cytoplasmic localization in response to 2% CSE for 4h. Actinmycin D (ActD) was used as positive control for the translocation of HuR. Lamin A/C is a nuclear marker, while Tubulin is a cytoplasmic marker. Results are expressed as the mean ± SEM of 4 independent experiments (2 different Normal subjects). **B.** Cytoplasmic HuR in COPD HLF: There was a significant increase in HuR cytoplasmic localization in response to 2% CSE for 4h (**p=0.001). Results are expressed as the mean ± SEM of 2 independent experiments (one COPD subject).

To confirm the localization of HuR by CSE, we used a complementary technique-imaging flow cytometry (ImageStream®). We observed a slight increase in the cytoplasmic localization of the HuR in Normal and COPD HLF exposed to 2% CSE for 4h (Figure 7A and 7B). Then, after gating the cytoplasmic fraction from the nuclear fraction, we quantify the intensity of the HuR in the cytoplasm by the software (IDEAS®). HuR intensity in the cytoplasm was slightly elevated in Normal and COPD HLF exposed to 2% CSE for 4h (Figure 7C). Collectively, these data indicate that cigarette smoke exposure induces the translocation of HuR into the cytoplasm.

A. Cytoplasmic HuR in Normal HLF- Imaging Flow Cytometry



B. Cytoplasmic HuR in COPD HLF- Imaging Flow Cytometry



C. Cytoplasmic HuR - HuR Intensity

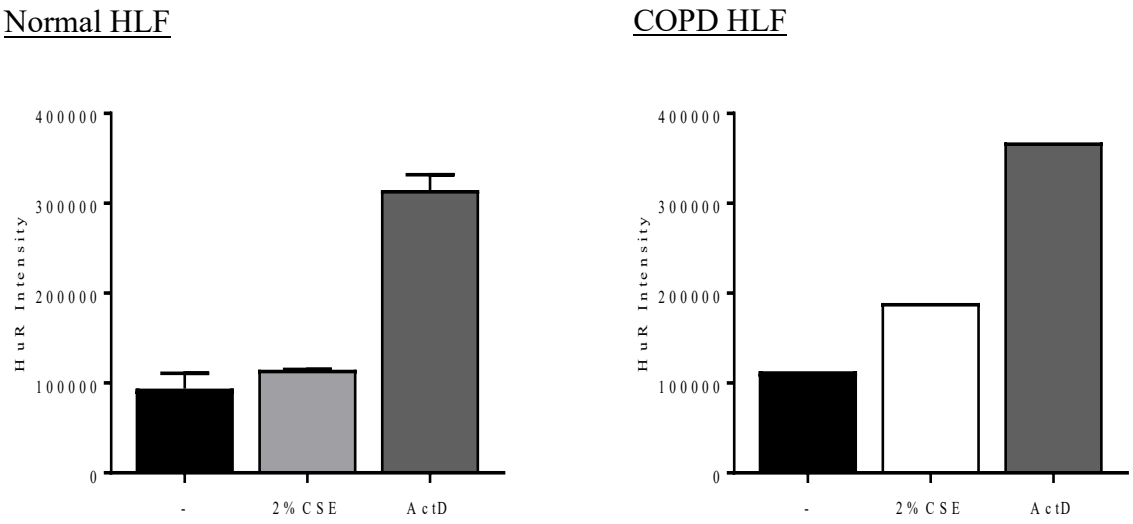
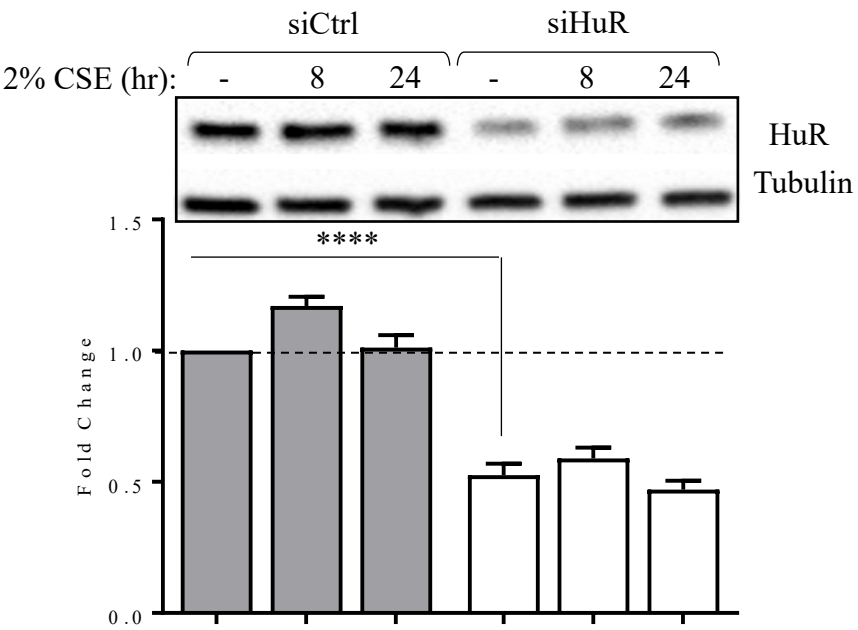


Figure 7. Cytoplasmic HuR protein levels increased in 2% CSE-exposed Normal and COPD HLF for four hours. A. Cytoplasmic HuR in Normal HLF- Imaging Flow Cytometry: HuR localization in Normal HLF treated with 2% CSE was assessed by Imaging Flow Cytometry. There was an increase in HuR expression in Normal HLF in response to 2% CSE for 4h. ActD was used as a positive control for HuR translocation into the cytoplasm. The nuclear expression of HuR in the untreated cells was not observed, because the permeabilization buffer was used in this experiment did not permeabilize nuclear envelope. A representative picture for cells is shown from 2 independent experiments (one Normal subject). **B.** Cytoplasmic HuR in COPD HLF- Imaging Flow Cytometry: There was an increase in HuR expression in the cytoplasm of COPD HLF exposed to 2% CSE for 4h. A representative picture for cells is shown from one experiment (one COPD subject). **C.** Cytoplasmic HuR - HuR Intensity: by using IDEAS® software, we quantify the intensity of the HuR in the cytoplasm of Normal and COPD HLF. There was an increase in the intensity of the HuR in the cytoplasm of Normal and COPD HLF exposed to 2% CSE for 4h by ~ 10,000 and ~70,000, respectively. Results are expressed as the mean ± SEM of 2 independent experiments from one Normal HLF and one experiment from COPD HLF.

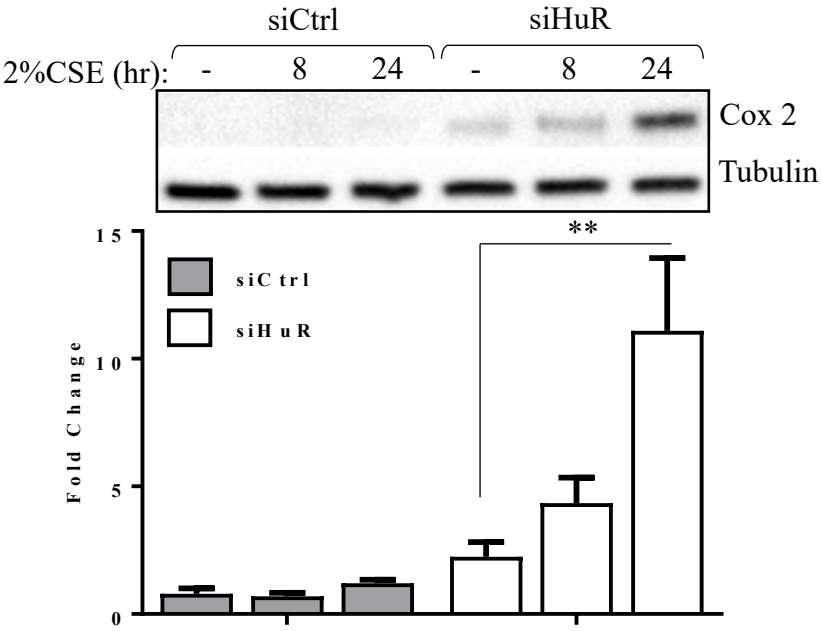
5.6 Knock-down of the HuR in Normal HLF increases the expression of COX-2 and IL-8.

COX-2 and IL-8 levels are elevated in COPD [11]. In HLF, COX-2 and IL-8 expression is increased in response to cigarette smoke [24, 25]. HuR stabilizes the mRNA of both *Cox-2* and *Il-8* [59]. Therefore, we speculated that HuR promotes smoke-induced expression of COX-2 and IL-8. To address this, we first transiently transfected Normal HLF with either HuR-specific siRNA (siHuR) or with control siRNA (siCtrl). Then, siCtrl and siHuR-transfected cells were treated with 2% CSE for 8h and 24h, and the supernatant and protein were collected. We confirmed that HuR levels were significantly reduced by 50% in the siHuR-transfected cells comparing to siCtrl-transfected cells (Figure 8A). Surprisingly, there was a significant increase in the level of COX-2 in siHuR-transfected cells treated with 2% CSE for 24h (Figure 8B). We also observed that IL-8 was significantly increased in response to 2% CSE for 24h only in the siHuR-transfected cells (Figure 8C).

A. siHuR- western blot- densitometry



B. COX-2 Protein- western blot- densitometry



C. IL-8 Protein- ELISA

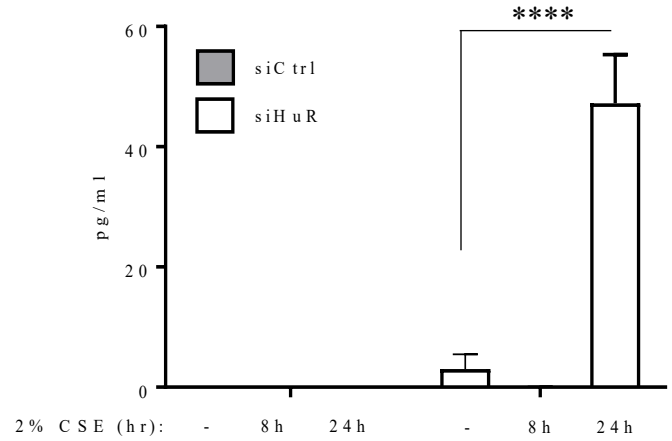


Figure 8. HuR silencing increases COX-2 and IL-8 protein expression in 2% CSE-exposed Normal HLF. A. siHuR- western blot- densitometry: transfection of Normal HLF with siHuR reduced the level of HuR protein to ~50%. Results are expressed as the mean ± SEM of 4 independent experiments (HLF used from one Normal subject). **B.** COX-2 Protein-densitometry: there was significant increase in COX-2 protein levels in siHuR-transfected HLF exposed to 2%CSE for 24h (**p= 0.001) compared to untreated siHuR-transfected cells and to siCtrl-transfected cells (***p= 0.0004). Results are expressed as the mean ± SEM of 4 independent experiments (HLF used from one Normal subject). **C.** IL-8 Protein- ELISA: there was significant increase in IL-8 protein levels in the media from siHuR-transfected HLF exposed to 2% CSE for 24h (****p=0.0001). Results are expressed as the mean ± SEM of 3 independent experiments (HLF used from one Normal subject).

We next evaluated the level of *cox-2* and *Il-8* mRNA in siHuR-transfected cells treated with 2% CSE for 24h. There was a robust increase in the level of *cox-2* and *Il-8* mRNA in siHuR-transfected cells treated with 2% CSE for 24h, as well as the untreated siHuR-transfected cells, compared to siCtrl-transfected cells (Figure 9A and 9B). These data indicate that knock-down of HuR increases COX-2 and IL-8 expression at the level of the protein and mRNAs.

A. *Cox-2* mRNA in siHuR- transfected cells **B.** *Il-8* mRNA in siHuR- transfected cells

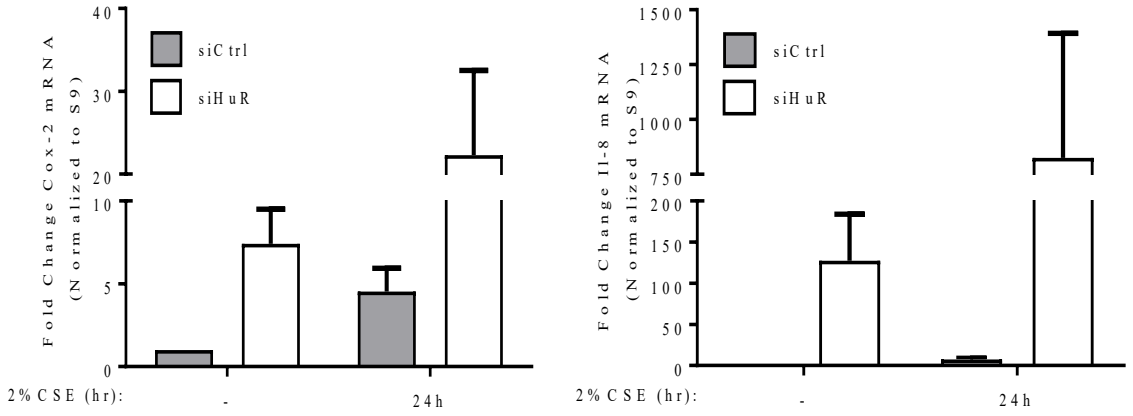
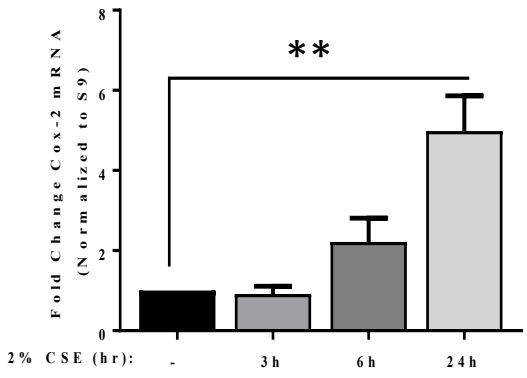


Figure 9. HuR silencing increases *Cox-2* and *Il-8* mRNA in 2% CSE-exposed Normal HLF. A. *Cox-2* mRNA in siHuR- transfected cell: There was an increase in the *Cox-2* mRNA in siHuR cells exposed to media only or 2% CSE for 24h. **B.** *Il-8* mRNA in siHuR- transfected cells: There was an increase in the *Il-8* mRNA in siHuR cells exposed to media only or 2% CSE for 24h. Results are expressed as the mean ± SEM of 2 independent experiments (HLF used from one Normal subject).

5.7 Knock-down of the HuR in Normal HLF does not affect the decay of *cox-2* and *Il-8* mRNAs.

Next, we assessed if HuR stabilizes *cox-2* and *Il-8* mRNA in response to 2% CSE. First, we treated Normal HLF with 2% CSE for 3h, 6h, and 24h to assess at which time point *cox-2* and *Il-8* mRNA is induced. We found that *cox-2* and *Il-8* mRNA were increased at 24h after exposure to 2% CSE (Figure 10A and B).

A. *Cox-2* mRNA



B. *Il-8* mRNA

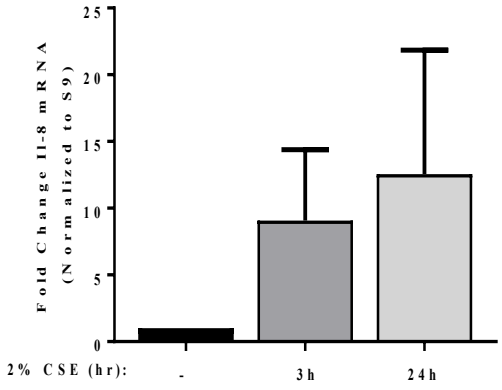


Figure 10. Cigarette smoke extract (CSE) increases *Cox-2* and *Il-8* mRNA in Normal HLF. A. *Cox-2* mRNA: There was a significant increase in the *Cox-2* mRNA in Normal HLF exposed to 2% CSE for 24h (*p< 0.05). Results are expressed as the mean ± SEM of 2-4 independent experiments (HLF used from one Normal subject). **B.** *Il-8* mRNA: There was an increase in the *Il-8* mRNA in Normal HLF exposed to 2%CSE for 24h. Results are expressed as the mean ± SEM of 2 independent experiments (HLF used from one Normal subject).

Then, we treated siCtrl and siHuR-transfected cells with 2% CSE for 24h followed by exposure to Actinomycin D treatment (ActD 1ug/ml) for ½ hour, 1h, 3h and 6h. We found that there was no significant difference in rates of *cox-2* and *Il-8* mRNA decay between siCtrl and siHuR transfected cells exposed to 2% CSE (Figure 11A and 11B). These data indicate that knock-down of HuR in Normal HLF did not affect the decay of *cox-2* and *Il-8* mRNA.

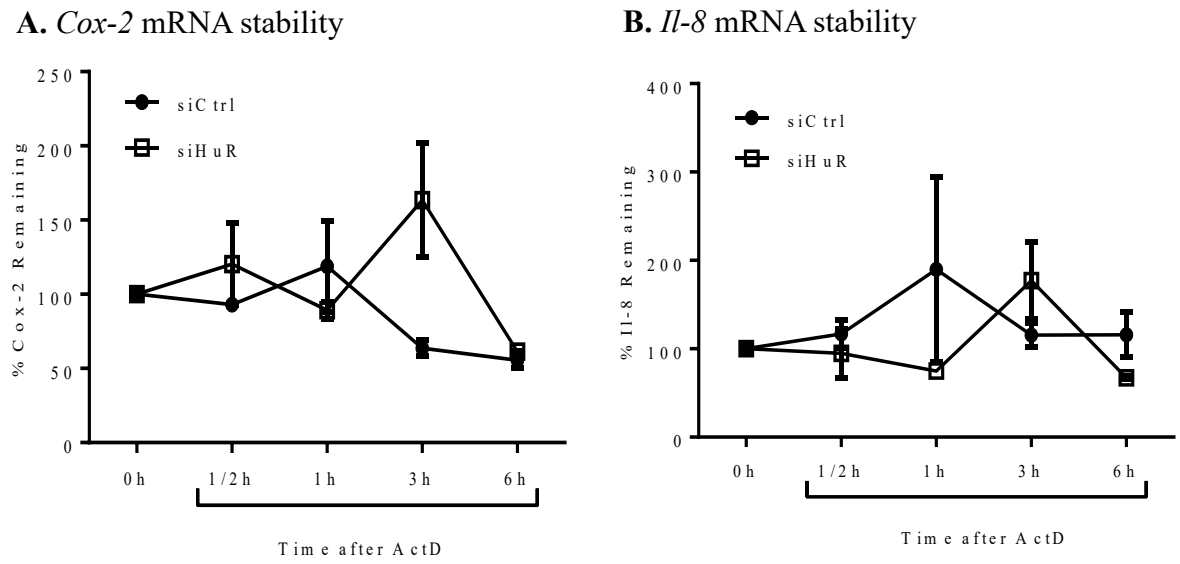


Figure 11. HuR silencing in Normal HLF does not affect the decay of *cox-2* and *Il-8* mRNAs A. *Cox-2* mRNA stability: siCtrl and siHuR transfected cells were treated with 2% CSE for 24h (*Cox-2* mRNA was induced at 24h in response to 2% CSE, see Figure 10) and then exposed to ActD (1 ug/ ml) for the indicated time point. *Cox-2* levels were set to equal 100% after CSE treatment for 24h and are expressed as percentage (%) of *Cox-2* mRNA remaining. In siCtrl and siHuR-transfected HLF, *Cox-2* mRNA was decreased after exposur to ActD for 6h compared to time 0. **B.** *Il-8* mRNA stability: siCtrl and siHuR transfected cells were treated with 2% CSE for 24h (*Il-8* mRNA was induced 24h in response to 2% CSE, see Figure 10) and then exposed to ActD (1 ug/ ml) for the indicated time points. *Il-8* levels were set to equal 100% after CSE treatment for 24h and are expressed as percentage (%) of *Il-8* mRNA remaining. In siCtrl and siHuR-transfected HLF, *Il-8* mRNA was remained stable after exposur to ActD for 6h compared to time 0. Results are expressed as the mean ± SEM of 2 independent experiments (HLF used from one Normal subject).

CHAPTER 6: DISCUSSION

COPD is a major health problem worldwide with limited therapeutic options. COPD is also a disease that develops primarily because of chronic cigarette smoke exposure [19, 86]. However, the mechanism(s) involved in cigarette smoke-induced lung damage are poorly understood. One of the main features of COPD is chronic inflammation, characterized by elevated levels of inflammatory protein mediators such as COX-2 and IL-8 [19, 24]. In this study, we focused on the link between COPD and the RNA-binding protein HuR. Previous studies have shown that HuR regulates the stability and/or the translation of target mRNAs that encode proteins involved in apoptosis, fibrosis and inflammation in response to stress [59, 61]. Therefore, depending on the stimuli applied to cells, the cellular function of HuR could vary [59]. For example, HuR stabilizes *Il-8* mRNA and induces its translation in response to IL-1β [80]. In addition, HuR has been implicated in lung cancer, which is another lung disease caused by cigarette smoke [77, 86]. High HuR expression in lung cancer tissue was associated with metastasis, poor survival and drug resistance [66, 78, 87]. One study revealed that targeting HuR by siRNA-based nanoparticles inhibited lung cancer cell proliferation, migration and invasion [88]. Thus, targeting HuR could be a novel and promising therapeutic strategy to treat smoke-related diseases such as lung cancer, but also COPD. We have previously shown that lung fibroblasts produce COX-2 in response to cigarette smoke [24, 81] and that the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor, destabilizes *Cox-2* mRNA by preventing HuR translocation into the cytoplasm [76]. These findings raise the possibility that the expression and the cytoplasmic translocation of HuR in lung cells may predispose to the aberrant inflammation in COPD, as well as in response to CS. In our current study, we sought to identify the expression and localization of HuR in Smoker and COPD subjects. We also report on the effect of CSE on the cytoplasmic translocation of HuR, which we predicted would regulate the expression of inflammatory proteins COX-2 and IL-8.

Because epithelial cells and fibroblasts are involved in the lung damage in response to CS via production of inflammatory mediators and proteases [11, 19, 26], we evaluated the expression and localization of HuR in these relevant lung cells. We observed that the cytoplasmic localization of HuR is increased in lung structural cells, mainly epithelial cells and fibroblasts in lung tissue from Smoker and COPD subjects. However, HuR remained predominantly in the nucleus of Normal lung tissue (Figure 1A). This suggests that smoking influences the cellular localization of HuR in the lung rather than caused by the disease itself. These findings are in contrast therefore with previous studies that there is elevated cytoplasmic HuR expression in several cancers, including lung, breast and colon cancer [77, 78, 89, 90]. A recent study revealed that cytoplasmic HuR expression was elevated in colonic epithelial cells from inflammatory bowel disease patients [91].

The increase in cytoplasmic localization of HuR is also associated with increased levels of inflammatory mediators [77, 79, 80]. For example, COX-2 expression in squamous cell carcinoma and non-small-cell lung carcinoma is associated with more cytoplasmic HuR [77, 78]. COX-2 over-expression was also associated with elevated cytoplasmic localization of HuR in colon cancer [92]. Another study revealed that *Il-8* mRNA binding to cytoplasmic HuR was increased upon exposure to IL-1β in breast cancer cells [80]. In parallel, COX-2 and IL-8 expression are also elevated in COPD and in response to CS [24, 38, 49, 51]. Altogether, these studies and our data suggest that increased cytoplasmic translocation of HuR in Smoker and COPD lung cells could be responsible to the high level of COX-2 and IL-8 expressed in COPD.

We also found that in macrophages from Smoker and COPD subjects, cytoplasmic localization of HuR is increased relative to macrophages from Normal subjects (Figure 1B). Macrophages number are increased in COPD and are involved in the pathophysiology of this disease by production of inflammatory mediators and proteases [26]. For example , it has been known that MMP-9 is increased in COPD, which is involved in alveolar destruction [19].

Interestingly, an independent study showed that in macrophages, HuR stabilizes *MMP-9* mRNA in response to β_2 integrin [93]. Furthermore, McMullen et al. revealed that chronic ethanol exposure enhances the translocation of HuR to the cytoplasm and its binding to *TNFα* mRNA in rat Kupffer cells (hepatic macrophages) [94]. Another group showed that human immunodeficiency virus (HIV) protease inhibitors, an antiviral drug that causes atherosclerosis, results in increased cytoplasmic expression of HuR, which stabilizes *TNF-α* and *Il-6* mRNA in macrophages [95]. These pro-inflammatory mediators are also increased in COPD [19]. Taken together, these studies and our data suggest that the elevated level of cytoplasmic HuR in macrophages from Smoker and COPD lung tissue could be responsible for the induction of pro-inflammatory mediators and proteases during COPD development.

Furthermore, *in vitro* analysis of the total HuR protein (full length HuR, 36 kDa) in HLF was similar between the three groups (Figure 2A and 2B). Although, we observed that cytoplasmic HuR was slightly, but not significantly, increased in Smoker HLF, its level was similar between COPD HLF and Normal HLF (Figure 2C and 2D). This data contradicts what we observed in lung fibroblasts from COPD subjects by mIHC (Figure 1A). We propose that the discrepancy between these two data could be a consequence of HuR protein cleavage. Indeed, it has been previously reported that in response to lethal cellular damage, HuR protein is cleaved by active caspase-3 into two fragments (CP-1, 27kDa and CP-2, 8kDa) [72, 96]. These HuR fragments involved in apoptosis and regulated the expression of proapoptotic mRNAs, such as *caspase-9* mRNA [72, 96, 97]. Previous studies have also reported that cleaved caspase-3 expression and apoptotic cell death are increased in COPD and in response to CS [98, 99]. Herein, we were unable to entirely validate this possibility by mIHC, because there is no specific antibody targeting cleaved HuR. However, we can detect cleaved HuR by western blot if we load at least 40 µg of protein extract [96, 100]. In this study, we observed only the full length HuR, but not the cleaved HuR, because we loaded 10 µg of protein extract.

Thus, it is possible that the elevated of HuR cytoplasmic expression observed in Smoker and COPD lung tissue could be a consequence of both the full length HuR and the cleaved HuR expression.

We next observed that exposure of Normal and COPD HLF to 2% CSE for 24h, slightly increased the total expression of HuR (Figure 3A, 3B and 3C). However, HuR translocation to the cytoplasm can lead to important functional changes in the cell. This supported by a report demonstrating that exposure to UV light did not significantly induce the total HuR expression, but it induced its translocation to the cytoplasm where stabilizes *p21* mRNA [101]. Nevertheless, nothing is known about the effect of cigarette smoke on the localization of HuR. Our data are the first to report that exposure to 2% CSE in Normal and COPD HLF results in increased HuR cytoplasmic translocation, which we confirmed using both western blot (Figure 6) and imaging flow cytometry (Figure 7).

Although the mechanism through which CSE-induced HuR cytoplasmic translocation is unknown, we propose that it may involve activation of p38 MAPK pathway. Previous and independent studies have illustrated that 1) cigarette smoke activates p38 [54, 102], and that 2) HuR phosphorylation by p38 MAPK results in alterations of HuR subcellular localization and/or its binding to target mRNAs [59]. Interestingly, phospho-p38 is elevated in epithelial cells and macrophages of COPD lung [102]. This raises the possibility that cigarette smoke-induced HuR cytoplasmic translocation may be mediated through p38 activation.

One of the more intriguing findings from our study is that when we knock-down HuR in Normal HLF and treated these cells with 2% CSE (Figure 8A), we observed an increase in COX-2 and IL-8 expression at both the protein and mRNA levels (Figure 8 and 9). However, this finding is not consistent with previous studies. First, one study demonstrated that knock-down of the HuR reduced COX-2, both at the protein and mRNA levels in response to UVB

[103]. Another study reported that knock-down of HuR decreased IL-8 secretion in primary human bronchial epithelial (HBE) cells exposed to 12% CSE in combination with human rhinovirus (HRV) [104]. One possible reason for the discrepancy between our results and those published in the literature may be due to the different treatment and/or cell type used. In this study, our data demonstrated that HuR silencing induced COX-2 and IL-8 expression in response to CS.

In support of our finding, one study performed in human Osteoarthritis (OA) chondrocytes observed that in response to IL-1β, *Cox-2* mRNA was sequestered in stress granules (SGs) by HuR. Thus, the protein level of COX-2 was decreased due to delay in the translation of *Cox-2* mRNA. SGs are cytoplasmic ribonucleoprotein complexes that assemble in response to stress and sequester mRNAs [105]. However, when OA chondrocytes transfected with siHuR, *Cox-2* mRNA did not translocate to SGs, and the protein level was elevated early upon IL-1β exposure [105]. In our study, we assume that CSE may induced SG assembly and HuR sequestered mRNAs in it, including *Cox-2* mRNA. This could explain why when we knock-down HuR we observed an increase in the level of COX-2 protein (Figure 8B). At this time, we do not know why there is elevated *Cox-2* mRNA upon HuR silencing or about SGs assembly during cigarette smoke exposure. These are areas of ongoing experimentation.

Alternatively, because HuR interacts cooperatively and competitively with other RBPs to stabilize target transcripts, it is possible that HuR silencing could increases activity and/or expression of other RBPs, which could lead to elevate COX-2 and IL-8 expression. For instance, HuR and TIA-1 stabilize mRNA encoding Programmed cell death 4 (PDCD4), which is a tumor suppressor. Wigington et al. found that increasing TIA-1 prevents HuR from binding to the *PDCD4* mRNA, while decreasing TIA-1 induces HuR binding to the *PDCD4* mRNA [62]. Nothing is known about the effect of HuR silencing on the expression of TIA-1 in response to CS. Moreover, it has been reported that HuR interacts with RBP called RNA

binding motif protein 3 (RBM3), which stabilizes *Cox-2* and *Il-8* mRNA [106, 107]. Both HuR and RBM3 expression have been reported to be elevated in cancer [106]. Another study revealed that co-expression of HuR and RBM3 in HCT116 (colorectal carcinoma cell line), increase the half-life of *Cox-2* and *Il-8* mRNAs up to 8h and 4h, respectively [107]. To our knowledge, nothing is known about the effect of HuR silencing or cigarette smoke exposure on the expression of RBM3.

We then postulated that HuR affects the stability of *Cox-2* and *Il-8* mRNAs in response to CSE. However, we observed that the knock-down of HuR had no effect on either *Cox-2* or *Il-8* mRNAs stability upon exposure to CSE (Figure 11A and 11B). This notion is supported by a study conducted in a mouse keratinocyte cell line, which observed that reduced HuR expression had no effect on UVB-induced *Cox-2* mRNA stabilization [108]. Additionally, another study showed that knock-down of HuR in human lung microvascular endothelial cells had no effect on *Il-8* mRNA stability after stimulation with TNF-α [109]. Altogether, our data and these studies suggest that HuR does not regulate the stability of *Cox-2* and *Il-8* mRNAs in these conditions.

Our work is the first to try to elucidate the link between HuR and CS- induced inflammation. However, one of the limitations of this study is the size of the lung tissue used for mIHC. These lung tissues were obtained from subjects that underwent lung resection surgery and only tissue from cancer-free regions were used. For this reason, the size of the lung tissue was small for some subjects and it was challenging to compare the results between the three groups.

Another limitation of our experiments is that our findings alone cannot definitively prove the role of HuR in inflammation in response to cigarette smoke. Despite these limitations, this study strongly supports the notion that HuR cytoplasmic translocation is elevated in

Smoker and COPD lung tissue. We also showed that CSE induces the cytoplasmic translocation of HuR in HLF, and that HuR regulates CS-induced inflammation. However, these data need to be confirmed in other models. In this context, it would be interesting to study the role of HuR in cigarette smoke-induced inflammation *in vivo*. Because HuR is known to target mRNA involved in organ development, complete deletion of the HuR, *in vivo* is lethal [110]. Thus, it would be worthwhile to conditionally knock-out the HuR in the mouse lung and then expose these mice and control mice to CS and assess the inflammation. In detail, conditional deletion of HuR in lung fibroblast of 8-week-old *Elavl 1*$^{fl/fl}$ *Col1α*2Cre mice via tamoxifen administration. However, knock-out of HuR in specific lung cell types, *in vivo,* such as epithelial cells and/or macrophages will be taken in consideration. In addition, we could conditionally knock-out the HuR in whole lung of *Elavl 1*$^{fl/fl}$ mice (8-week-old) by using intranasal (IN) instillation of adenovirus-mediated Cre recombinase. Furthermore, and from a clinical perspective, HuR cytoplasmic localization in lung structural cells and inflammatory cells from Smoker and COPD subjects could be compared to Normal subjects. This strategy may be useful as a biomarker for susceptibility to COPD.

In conclusion, we are the first to report the expression and localization of HuR in human lung tissue from Smoker and COPD, and show that cigarette smoke induces HuR translocation to the cytoplasm in human lung fibroblasts. Our data also show that knock-down of HuR in Normal human lung fibroblast increases the expression of COX-2 and IL-8 in response to *in vitro* exposure of cigarette smoke extract. We also show that knock-down of HuR has no effect on either *Cox-2* or *Il-8* mRNAs stability in response to CSE. Further molecular investigation into the post-translational modifications of HuR will improve our understanding of HuR in COPD and may contribute to the development of novel target therapy for smoke-related diseases such as COPD.

CHAPTER 7: REFERENCES

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