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THE ROLE OF SRC KINASE ACTIVATION IN LUNG EPITHELIAL ALTERATIONS IN RESPONSE TO THE α,β-UNSATURATED ALDEHYDE ACROLEIN

A Thesis Presented

by

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of

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In Partial Fulfillment of the Requirements for the Degree of Master of Science Specializing in Cellular, Molecular, and Biomedical Sciences

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ABSTRACT

Cigarette smoke (CS) exposure is the leading cause of preventable death in the United States contributing to over 480,000 deaths a year with over 300 billion dollars in CS related costs spent per year. While the dangers of CS exposure have been studied and characterized for decades being largely attributed to reactive oxygen species and oxidative stress, increasing evidence suggests that reactive aldehydes in CS, specifically the α,β-unsaturated aldehyde acrolein, are responsible for many of the negative pathologies associated CS exposure. Previous work has shown that acrolein can bind directly to a number of cellular proteins containing redox sensitive cysteine residues. The non-receptor tyrosine kinase Src contains nine cysteine residues and is known to be activated in response to CS and oxidative stress. Despite being the first characterized and one of the most widely studied oncogenes, the exact mechanism for Src activation remains unclear. In the current studies we examined the effects of acrolein on Src activation and the resulting outcomes on the lung epithelium in an effort to better understand how reactive electrophiles in CS contribute to the development of lung disease.

To determine the effects of acrolein on Src activation, we first exposed NCI-H292 cells to acrolein and measured activity by western blot. We observed an increase in Src activity detected by an increase in Src phosphorylation at Y416 and an increase in phosphorylation of Src target proteins Caveolin1 and p120. Interestingly the increase in activation occurred without dephosphorylation of the inhibitory phosphorylated tyrosine Y527. Using biochemical-labeling strategies we identified Src as a direct target of acrolein adduction in vitro and in vivo, and we used mass spectrometry to confirm acrolein adduction to cysteine residues C245, C277 and C487, all which have been implicated in a redox dependent Src activation mechanism. Furthermore, increased Src activity following acrolein exposure was confirmed using an in vitro kinase activity assay and recombinant Src in a cell free system.

To study the effects of acute acrolein exposure on lung epithelial function we exposed cultured mouse tracheal epithelial cells (MTECs) to acrolein and show impaired epithelial barrier function, measured by a decrease in trans epithelial resistance (TER) and increased epithelial permeability to FITC-dextran, which could be prevented using the Src inhibitor PP2. Src inhibition also attenuated acrolein-induced loss of E-cadherin and ZO-1. Acute exposure of C57BL/6 mice to acrolein (5 ppm for 4 hrs) led to increased epithelial permeability, measured by enhanced leakage of i.v. injected FITC-dextran into the airspaces, and induction of HO-1 in the lung while chronic acrolein exposure resulted features of epithelial to mesenchymal transition including a reduction of E-cadherin, increased vimentin, increased expression of MMP9 and increased collagen deposition. Chronic acrolein exposure in vitro resulted in a reduction of E-cadherin that could be prevented using the Src inhibitor AZD0530.

Together our studies demonstrate that Src is a direct target for acrolein and plays an important role in epithelial alterations due to acrolein exposure. This work provides further insight into a potential mechanism involved in the development of cigarette smoke related disease and could provide a potential target for novel therapeutics.
ACKNOWLEDGEMENTS

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CHAPTER 1: LITERATURE REVIEW

1.1 Cigarette smoke

Over 480,000 people die every year as a result of cigarette smoke (CS) exposure making smoking the 3rd leading cause of preventable death in the United States with associated health care costs rising as high as 300 billion dollars spent per year [1]. Exposure to cigarette smoke increases the risk of developing lung cancer and COPD contributing to 90% of lung cancer related deaths and 80% of COPD related deaths [1]. Additionally, childhood exposure to CS is the only proven cause of asthma and can also result in worsening of asthma symptoms [2].

Over 5,000 chemicals are produced through burning tobacco and interact with the lung epithelium in a solid phase and gaseous phase. Past research has attributed the negative effects of CS to the presence of reactive oxygen species (ROS) and increased oxidative stress within the cell. It’s been shown that CS induces DNA damage due to oxidative stress and that patients with COPD show an increase in protein oxidation in the lung compared to healthy lungs [3]. Additionally, the oxidative stress induced by CS is associated with an increase in the development of lung cancer [4]. Cigarette smoke exposure affects cellular systems in a variety of ways having both immunosuppressive and pro-inflammatory properties. CS impairs the innate immune response to allergens by inhibiting T-cell activation and impairing macrophage phagocytosis [5]. Exposure to cigarette smoke exposure also contributes to COPD and emphysema by repeatedly stimulating pro-inflammatory pathways and by promoting an increase in apoptosis
involving the generation of ceramide or by increasing cellular senescence involving the proteins p53 and p16 [6, 7]. Some of the effects of cigarette smoke on the lung epithelium are summarized in Figure 1.1 below.

![Figure 1.1 Effects of cigarette smoke on the lung epithelium.](image)

To better understand the effects of cigarette smoke, single components are often studied to determine their individual effects on cellular responses. It was determined that acrolein and crotonaldehyde were the primary glutathione (GSH) adducts formed in lung epithelial cells following CS smoke exposure indicating that acrolein alters the availability of thiol based antioxidants and may be linked to signaling pathways involving protein oxidation by reactive oxygen species [8]. Indeed, acrolein is known to elicit many
of the same cellular responses as CS such as increased mucus metaplasia, collagen deposition, NF-κB activation, irritation to the respiratory tract, and altered host defense, and may be a primary contributing factor to the underlying signaling mechanisms involved in CS exposure[9, 10]. One classification scheme has separated chemicals of CS into two groups consisting of cancer causing and non-cancer hazards. Of the non-cancer hazards found in CS, acrolein was the most abundant chemical identified [11].

1.2 Acrolein

The α,β-unsaturated aldehyde acrolein (2-propenal) is the smallest unsaturated aldehyde and is produced through the incomplete combustion of organic matter including fossil fuels, wood stoves, and cooking oil [12]. The largest source of acrolein exposure that poses the greatest risk to human health is through the inhalation of cigarette smoke with concentrations in mainstream smoke ranging as high as 90 ppm, which is significant because levels as low as 0.5 ppm cause lacrimation while concentrations of 5.5 ppm cause irritation to the eyes and nose [13]. Using HPLC it was estimated that cigarette smoke exposure resulted in acrolein concentrations as high as 80 μM in respiratory tract lining fluid [14]. Acrolein exposure has been associated with cell signaling pathways involved in the development of COPD and is measured to be higher in the lungs of patients with asthma and COPD [15]. Acrolein is seen to mimic many of the same effects as CS including a suppressed innate macrophage response, suppressed host defense, and increased mucus hypersecretion, suggesting that acrolein is a major contributor to CS related pathologies [16-18].
Acrolein is classified as a soft electrophile based on the ability of its surrounding electron cloud to be highly polarizable creating an electron dense region near its carbonyl with an electron deficient region at its terminal β-carbon [19]. As a soft electrophile, acrolein primarily reacts with soft nucleophiles such as cysteine, lysine and histidine residues through a process known as Michael addition occurring though its terminal β-carbon (Fig 1.2A).

![Diagram](image)

**Figure 1.2 Formation of cysteine to lysine cross links through acrolein.**

The cysteine sulfhydryl thiolate is one of the softest biological nucleophiles making it one of the most prominent targets of acrolein [19]. Previous work in the van der Vliet laboratory has shown through mass spectrometry that the majority of acrolein adducts in the cell occur on cysteine residues [20]. The carbonyl of acrolein is also reactive with the ability to form a Schiff base with the terminal amine of lysine residues and the DNA base deoxyguanosine [21]. The two reactive sites on acrolein allow for the formation of protein-protein cross-links through Michael addition to cysteine residues and subsequent Schiff base formation with lysine. If a cross link forms the subsequent separation of the two residues results in a unique +38.01565 Da adduct present on the
lysine residue with regeneration of free cysteine that is detectable using mass spectrometry (Fig 1.2B) [22].

One of the main detoxification mechanisms of acrolein is through conjugation with glutathione (GSH), which can occur with or without the use of a catalyst or enzyme [12]. Acrolein exposure has been shown to rapidly deplete cellular levels of GSH that are recoverable over time partially through increased synthesis of GSH [23]. Previous work in the van der Vliet laboratory has also shown that GSH plays a role in reversing protein alkylation demonstrated by the presence of more acrolein adducts in cells treated with the GSH synthesis inhibitor BSO and that reversal of acrolein adducts is not dependent on the actions of the 26S proteasome [23]. Additionally, mice expressing the enzyme glutathione S-transferase P (GSTP) showed fewer acrolein adducts over time compared to GSTP null mice suggesting that GSTP plays a role in the reversal of acrolein-protein adducts by promoting the adducts transfer and conjugation with GSH [24]. This has important implications for the use of electrophiles as a form of reversible signaling mechanism in the cell.

Once acrolein is bound to GSH the conjugate begins degradation starting primarily in the liver where acrolein-bound GSH undergoes cleavage of its γ-glutamic acid then subsequent cleavage of glycine. Upon reaching the kidney the conjugate is N-acetylated to form the molecule S-(3-oxopropyl)-N-acetylcysteine [25]. The aldehyde present on the molecule can then undergo reduction to form S-(3-hydroxy-propyl)-N-acetylcysteine or oxidation to form S-carboxyethyl-N-acetylcysteine that is then excreted and detectable as acrolein metabolites in urine [26].
Detoxification of acrolein can also occur without the conjugation to proteins or antioxidants. Oxidation of acrolein by aldehyde dehydrogenase results in the generation of acrylic acid, 44-80% of which is metabolized to carbon dioxide as seen in studies using heavy acrylic acid administered to rats [12, 27, 28]. After acrolein binds to cysteine residues it can create cross-links with lysine by forming a Schiff base with lysine’s ε-amino group as described before. This is only an intermediate species that undergoes an electron shift rearrangement resulting in the regeneration of free cysteine thiol.

In summary, studying the effects of acrolein on the lung epithelium will provide further insight into understanding the signaling mechanisms and hazards associated with CS exposure. Although there are thousands of chemicals present in CS, acrolein is shown to be a key mediator of many CS related outcomes and remains an important molecule of study. Further research is necessary to better understand how acrolein reacts with proteins and signaling pathways in the lung epithelium and how these alterations affect normal lung biology.

1.3. Src

The non-receptor tyrosine kinase Src is the prototypical member of the Src family kinases and is ubiquitously expressed in the body. Src was first discovered following research established by Peyton Rous, showing that a viral infection could produce tumors in chickens [29]. Identification of Src as the first proto-oncogene led to Nobel Prizes in 1996 and 1989. Overexpression of Src is commonly observed in cancers with Src activation promoting increased cell migration, invasiveness, and epithelial remodeling.
A few of the effects of cigarette smoke on the lung epithelium are summarized in Figure 1.3.

<table>
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<td>↑ pulmonary fibrosis in response to TGF-β1 (Hu et al 2014)</td>
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<td>↑ EGF receptor activation (Sham et al 2013)</td>
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<td>Src inhibition can prevent CS induced EMT and loss of epithelial barrier function (Zhang et al 2012)</td>
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**Figure 2.3 Effects of Src activation on the lung epithelium**

There are nine different Src family kinases that share a conserved sequence homology consisting primarily of three different domains, those being an SH2 domain, an SH3 domain and a kinase domain. Src also contains a C-terminal regulatory tail domain and a unique SH4 domain at the N-terminus that includes a myristoyl group that can insert into the plasma membrane and assists in sub cellular localization [31]. Under basal conditions Src is held in an inactive conformation through interactions between its SH2 domain and a phosphorylated tyrosine residue (Y527) on the C-terminal tail as well as interactions between its SH3 domain and a proline P-X-X-P consensus sequence found between the SH3 domain and the kinase domain [32]. These interactions maintain Src in a closed inactive conformation. Regulation of Src activity classically involves the dephosphorylation of Y527 that allows the protein to unfold and undergo a conformational change. Once open, Src autophosphorylates at Y416 further increasing
kinase activity that can be detected by western blot as a measure of kinase activation [33]. Tyrosine Y416 is located on a flexible alpha-helix loop (A-loop) on the kinase domain and takes on an ordered conformation under basal conditions and adds to the negative regulation of Src activity by filling the catalytic cleft of the kinase domain restricting access to ATP and substrates [34].

Viral Src (v-Src) was identified as the responsible factor present in the Raus sarcoma virus that let to its transforming ability. Sequence analysis revealed that v-Src differed from cellular Src in that it lacked the C-terminal regulatory tail domain containing Y527 [34]. Since a closed inactive conformation could not be maintained through binding interactions between the C-terminal tail and the SH2 domain, it was found that v-Src was constitutively active. As a result, cells containing v-Src show increased invasiveness, proliferation, and resistance to apoptosis [31].

Phosphorylation of Src at Y527 is regulated through the dynamic activity between kinases and phosphatases. Phosphorylation is decreased by protein tyrosine phosphatases such as SHP1 and PTP1B, leading to a release of the SH2 domain and opening of the protein resulting in increased kinase activity. The catalytic site of PTP1B contains a redox sensitive cysteine residue and has been shown to be inactivated through oxidation or through adduction to acrolein [35]. Phosphorylation of Y527 is performed by Csk (c-Src kinase) promoting the inactive closed form of Src. Additional regulation by Csk occurs through the protein Cbp (Csk binding protein), which is a transmembrane protein and recruits Csk to the membrane where Src is localized promoting their interaction and phosphorylation [36]. The only phosphatase known to remove phosphate from Y416 is
PTP-BAS [33]. Alteration of activity in these different kinases and phosphatases are commonly found in diseased states associated with altered Src activity [37].

Recent studies have shown that Src can also be regulated through the oxidation of cysteine residues located throughout the protein. Src contains 9 cysteine residues, 3 in the SH2 domain and 6 in the kinase domain. Through the generation of mutant cell lines containing cysteine to alanine mutations, Giannoni et al. 2005 demonstrated that the oxidation of cysteine residues C245 and C487 are involved in an oxidant induced activation of kinase activity, and suggest the oxidation events result in the formation of an intramolecular disulfide bond [38]. The importance of cysteine oxidation in activation of kinase activity was further supported by Zhang et al. 2014, providing evidence of a redox based activation mechanism involving oxidation of cysteine residues C245, C277, and C501 in response to TGFβ1 [39]. Interestingly, contrasting evidence supports the inactivation of Src activity in response to oxidants. Kemble et al. 2008 showed that oxidation of C277 results in the formation of a Src dimer through an intermolecular disulfide bond that inactivates kinase activity [40]. In addition to activation by oxidation, Src can be activated by the lipid peroxidation product 4-Hydroxynonenal through the mediation of PTP1B, and EGFR signaling [41].

Exposure to CS results in the activation of Src, however the exact activation mechanism remains unclear. Chronic cigarette smoke exposure causes remodeling of the lung epithelium with an increase in expression of MMP9, increased MAPK signaling and increased lung tissue destruction, mediated through activation of Src [42]. Additionally, features of EMT such as loss of E-cadherin, gain of vimentin and smooth muscle actin
occur through Src mediated pathways in response to CS exposure [43]. CS exposure weakens the lung epithelium through the disruption of cell contacts known as adherens junctions [44]. E-cadherin is the primary structural component of adherens junctions and links the cell membrane to the actin cytoskeleton. Src regulates the stability of E-cadherin through the phosphorylation of the adherens junction protein p120, where upon phosphorylation it dissociates from the adherens junction complex resulting in E-cadherin endocytosis and degradation [45].

Current research has established Src as a key protein that is activated in response to cigarette smoke, however the mechanism of activation remains uncertain. While oxidative signaling mechanisms have been proposed, we looked to determine the effects of acrolein on Src activation and how Src activation plays a role in the effects of acrolein on lung epithelial cells.

1.4 Current Studies

Our primary goal of this work was to assess Src activation in response to acrolein and the role of Src in acute and chronic acrolein exposure in the lung epithelium. We hypothesize that chronic acrolein exposure will result in features of EMT and lung remodeling similar to those observed by chronic cigarette smoke exposure. Additionally, we hypothesized that acrolein activates Src by directly modifying cysteine residues on the protein to promote an active conformation. To test our first hypothesis we measured the effects of acute and chronic acrolein exposure in mouse tracheal epithelial cells and in an in vivo mouse exposure model. We utilized the Src inhibitors PP2 and AZD0530 to test to role of Src in these responses. To test our second hypothesis we exposed H292 cells or
purified Src to acrolein and measured activation through detection of Src targets using western blot or an *in vitro* kinase activity assay. Additionally, we used biotin conjugated probes and mass spectrometry to identify cysteine modification in H292 cells and recombinant Src respectively.
1.5 References


32. Boerner, R.J., et al., *Correlation of the phosphorylation states of pp60c-src with tyrosine kinase activity: the intramolecular pY530-SH2 complex retains
CHAPTER 2: ACROLEIN REDUCES EPITHELIAL BARRIER FUNCTION THROUGH SRC ACTIVATION

2.1. Abstract

The lung epithelium provides the first line of defense against inhaled chemicals, pathogens, and particulate matter through the physical barrier it creates between the lung and the external environment. It has been observed that acute cigarette smoke exposure leads to a weakening of epithelial barrier function and a loss of adherens junction proteins, whereas chronic cigarette smoke exposure leads to tissue destruction and a phenomenon known as Epithelial to Mesenchymal Transition (EMT) through activation of the Src kinase. In the current studies we tested the effects of acrolein, a highly reactive chemical found in cigarette smoke, on the lung epithelium in both acute and chronic exposure settings and examined the role of Src activation in these responses. Acute acrolein exposure of mouse tracheal epithelial cells (MTECs) resulted in a dose dependent decrease in barrier resistance measured by trans epithelial resistance (TER) and a subsequent increase in epithelial permeability to FITC-dextran. Pretreatment with the Src inhibitor PP2 prevented loss of epithelial barrier function. Further analysis of protein by western blot revealed a dose dependent loss of junction proteins E-cadherin and ZO-1, which was prevented with Src inhibition. Using confocal microscopy we show that E-cadherin is lost from the cell membrane while simultaneous studies using biotin conjugated probes show a loss of E-cadherin surface labeling following acrolein exposure. Preliminary studies show that acute acrolein exposure of C57BL/6 mice (5
ppm for 4 hrs) resulted in increased airway epithelial permeability that could be attenuated using the Src inhibitor AZD0530. Together these studies show that acrolein exposure causes a weakening of the epithelial through loss of E-cadherin in a Src mediated pathway.
2.2. Introduction

Chronic obstructive pulmonary disease (COPD) is a group of diseases characterized by an irreversible restriction of airflow in the lung and includes the disorders emphysema and chronic bronchitis. COPD differs from asthma in that inflammation and restriction of airways is not reversible. In patients with COPD, chronic inflammation, fibrosis and mucus secretion leads to a destruction of lung tissue and loss of lung function [46]. Smoking cigarettes is one of the greatest risk factors for the development of COPD with over half of COPD patients being smokers [1]. It’s been shown that reactive oxygen species (ROS) generated in CS contribute to the development of COPD by increasing activation of the non-receptor tyrosine kinase Src and Epidermal Growth Factor Receptor (EGFR) partially through MAPK signaling leading to epithelial injury [6]. Activation of Src has largely been studied as a contributing factor in the development of cancer, being the first characterized oncogene contributing to increased cell migration, proliferation, and invasion, although increasing evidence suggests these properties of Src activation are also involved in the development of COPD [6, 31]. Stability of the adherens junction protein E-cadherin is regulated by Src activation and is decreased in response to CS leading to weakened epithelial barrier function, which is a feature of COPD [44, 47]. The alpha beta unsaturated aldehyde acrolein is present in cigarette smoke at levels as high as 90 ppm and has been shown to cause many of the negative effects of CS [13].

The lung epithelium protects the body from inhaled particulate matter, pathogens and environmental pollutants by providing a physical barrier that is maintained through
cell-to-cell connections known tight junctions and adherens junctions [48]. The adherens junction is made up of a complex of proteins including E-cadherin, alpha catenin, beta catenin, and delta catenin also known as p120. E-cadherin is a transmembrane protein that forms the connection to neighboring cells through its extracellular Ca+ binding domain while its intracellular domain links the cell membrane to the actin cytoskeleton through alpha and beta catenin and p120 [49]. p120 stabilizes the adherens junction through binding to E-cadherin and is a phosphorylation target of Src [50]. Upon phosphorylation of tyrosine Y228, p120 dissociates from the adherens junction increasing E-cadherin endocytosis and degradation. Additionally p120 expression is observed to be decreased or absent in a number of cancers suggesting that the association of p120 with E-cadherin could act as a tumor suppressor by maintaining a proper epithelial cell like phenotype [51].

Loss of E-cadherin is commonly associated with the development and progression of cancer involving a process known as epithelial to mesenchymal transition (EMT). Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells lose their epithelial phenotype and gain a more mesenchymal phenotype including enhanced cell migration, invasiveness, and increased metastatic potential [52]. One of the hallmark characteristics of EMT is the loss of epithelial cell markers E-cadherin and a gain of mesenchymal gene expression of proteins such as vimentin, fibronectin, and smooth muscle actin. Previous research has shown that chronic cigarette smoke exposure results in the development of EMT through the activation of Src [42, 43]. In the current studies we examined the effects of chronic acrolein exposure on the lung epithelium to see if
acrolein could mimic the effects of chronic CS exposure such as a loss of E-cadherin, increased collagen deposition, and other features of EMT.

We tested the effects of acute and chronic acrolein exposure on the lung epithelium in both *in vitro* and *in vivo* models and studied how Src activation regulates airway epithelial injury and barrier integrity through control of E-cadherin stability and endocytosis. We hypothesized that acute acrolein exposure causes lung epithelial damage through Src activation while chronic acrolein exposure leads to lung remodeling events similar to those following chronic cigarette smoke exposure. Our findings demonstrate that acrolein reduces epithelial barrier integrity that can be prevented through Src inhibition. We show that this process occurs through the activation of Src and subsequent degradation of E-cadherin.
2.3. Methods

2.3.1. Cell Culture

NCI-H292 cells (ATCC) were cultured in RPMI-1640 (Gibco) media supplemented with 10% FBS and 1% Penicillin/Streptomycin (Gibco). Primary mouse tracheal epithelial cells (MTECs) were isolated from C57BL/6J mice (Charles River) as previously described by Wu and Smith [53]. MTECs were grown in DMEM/F12 media supplemented with 20 ng/mL cholera toxin (List Biological Laboratories; Campbell, CA), 5 μg/mL insulin (Sigma), 5 μg/mL transferrin (Sigma), 15 μg/mL bovine pituitary extract (Invitrogen), 10 ng/mL EGF (Calbiochem; San Diego, CA), 100 nM dexamethasone (Sigma), 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. Cells were grown in a 5% CO$_2$ environment at 37°C.

2.3.2. Cell Treatments

Cells were starved in serum free media without phenol red for 24 hours prior to experimentation. Starvation media was replaced at least one hour prior to experiment. Acrolein (Sigma) was diluted in H$_2$O to a working concentration of 10 mM then added to media for final concentrations ranging from 1-100 μM. Inhibitors PP2 and AZD0530 (Saracatinib) were reconstituted in DMSO for a stock solution of 10 mM then diluted in serum free media without phenol red to a concentration of 10 μM PP2 or 1 μM AZD0530. Cells were incubated with inhibitors for 30 min prior to experiment. For chronic acrolein exposure in MTECs, cells were treated with 3, 10, or 30 μM acrolein every day, while media was changed every other day. Following acrolein treatment, cells were washed once with cold PBS before being lysed. To collect protein cells were lysed
on ice for 30 minutes in Western Solubilization Buffer (WSB) containing 1% Triton, 50 mM HEPES, 250 mM NaCl, 10% Glycerol, 1.5 mM MgCl₂, 1 mM PMSF, 1 mM EGTA, 2 mM Na₃VO₄, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin. Protein levels were determined using a bicinchoninic acid (BCA) protein assay (Pierce). To collect RNA cells were lysed in buffer supplied by the Gene Jet RNA Purification Kit (Thermo) following the manufacturers protocol.

2.3.3. MTEC permeability assay

MTECs were seeded at 200,000 cells/well in 12-well Transwell® inserts (Corning) that were coated in 50 µg/ml rat tail collagen-1, and cultured in full media as previously described. Cells were grown until tight junctions had formed, which was determined by a plateau in trans-epithelial resistance (TER) measured by a volt-ohm meter (World Precision Instruments). Prior to acrolein exposure, designated cells were treated apically with 10 µM PP2 in full media. Acrolein was prepared as previously described and added to inserts at final concentrations of 30 and 100 µM. TER was measured over the course of 24 hours at which point apical media was removed and replaced with phenol free media containing 5 mg/mL FITC-Dextran (Sigma, molecular weight 4kDa). After an additional 24 hours fluorescence of basal media was measured (excitation 492 nm; emission 520 nm) using a Biotek Synergy HT plate reader (BioTek). Cells were then washed twice with cold PBS and lysed with Western Solubilization Buffer (WSB) for protein analysis.

2.3.4. Western blot
Equal amounts of protein were loaded in 10% tris-glycine SDS-PAGE gels (BioRad), separated and transferred to a nitrocellulose membrane (Thermo), and blocked in 5% non-fat milk. Primary antibodies for Src, pSrc-Y416, E-cadherin, p120, pp120-Y936, were supplied by Cell Signaling Technologies and used at a dilution of 1:1000 in 5% BSA in tris-buffered saline plus 0.1% Tween® 20 (TBS-T). Beta-actin (Sigma) was used at a dilution of 1:10,000 in TBS-T 5% BSA. Membranes were incubated with secondary HRP conjugated rabbit and mouse antibodies (Cell Signaling) at a dilution of 1:1000 in 5% milk in TBS-T. Proteins were detected with enhanced chemiluminescent substrate (Pierce) and x-ray film (Kodak) or the Amersham™ Imager 600 (GE Life Sciences).

2.3.5. In vivo acrolein exposure

For an acute acrolein exposure in vivo, C57BL/6 mice (Charles River) were exposed to 5 ppm acrolein (Sigma) vapor at a flow rate of 2.5 L/min for 4 hours in a glass exposure chamber. Acrolein concentration was monitored by a Miran SapphIRe air analyzer (Thermo Scientific). Immediately after acute exposure, mice were sacrificed to harvest lung tissue for protein and RNA analysis. Mice were also submitted to a chronic exposure of 5 ppm acrolein for 4 hours, 5 days/week for 2 weeks. In the chronic exposure model, mice were sacrificed 3 days after the last acrolein exposure to harvest bronchoalveolar lavage fluid (BALF), protein and RNA. After trachea cannulation, lungs were washed three times using 500 µL sterile PBS as room temperature. After spinning at 1,500 rpm for 5 min to pellet cells, lavage fluid was removed for analysis by ELISA, while the cell pellet was resuspended in 500 µL PBS and split for analysis by an ADVIA
hematology system (Siemens) (200 µL) and cytospin (300 µL). Cytospin slides were stained with hematoxylin and eosin.

2.3.6. FITC-dextran injections

Prior to acrolein exposure mice were given vehicle (PBS) or the Src inhibitor AZD0530 at a concentration of 50 nM in 50 µL through nasal instillation. Mice were then injected with 25 mg/mL FITC-Dextran in the tail vein and exposed to 5 ppm acrolein for 4 hours. BALF was collected by lung lavage two times with 500 µL PBS at room temperature. Blood was collected with a pipette after severing the carotid artery and spun down at 8,000 rpm in serum collection tubes (BD Microtainer). BALF and serum fluorescence was measured (excitation 492 nm; emission 520 nm) using a Biotek Synergy HT plate reader (BioTek). Epithelial permeability was measured as the ratio of BALF to serum fluorescence.

2.3.7. Confocal Microscopy

H292 cells were seeded in glass chamber slides (Millipore) and grown until >90% confluent. Acrolein (Sigma) was prepared as described above and added to wells at a concentration of 100 µM for 3 hours. Cells were washed once with cold PBS then fixed with 4% paraformaldehyde (PFA) in 1% bovine serum albumin (BSA) for 1 hour. Cells were permeabilized with 0.2% TritonX100 in 1% BSA for 15 min then blocked with 10% goat serum for 1 hour. Cells were stained with an E-cadherin rabbit primary antibody (1:250) and secondary goat anti-rabbit Alexa-555 conjugated IgG (1:500). Cell nuclei were stained with 10 µg/mL DAPI, and then imaged using a Zeiss LSM confocal microscope at 25X magnification.
2.3.8. Transfections

H292 cells were transfected at 50% confluence in serum free media using SMARTpool Src siRNA (Dharmacon) and DharmaFECT™ transfection reagent (Dharmacon) overnight. Transfected cells were then grown in serum containing media for 24 hours followed by starvation and experimentation.

2.3.9. PCR

RNA was isolated from cell lysates or mouse lung homogenates using the GeneJET RNA purification kit (Thermo) as directed by the manufacturer and quantified using a Biotek plate reader. cDNA was generated using Oligo(dT) 12-16 primer (Promega), dNTPs (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). qRT-PCR was performed using SYBR Green Supermix (BioRad) and normalized to GAPDH.

Primer Sequences:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CTGGGAGAAACCTGCCAAGTA</td>
<td>TGTTGCTGTAGCCGTATTCA</td>
</tr>
<tr>
<td>HO-1</td>
<td>AAGACTGCGTTCCCTGGTCAAC</td>
<td>AAAGCCCTACAGCAACTGTGC</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>TCTTGCCGTTTCTTTCAAC</td>
<td>CAAAGATCCAGCCAGAAAA</td>
</tr>
<tr>
<td>Vimentin</td>
<td>TCCAAGTTTGCTGACCTCTCTC</td>
<td>CTCTTCCATCTCAGCATC</td>
</tr>
<tr>
<td>TGFB1</td>
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<td>MMP9</td>
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<tr>
<td>Collagen1A1</td>
<td>CACCCCTCAAGAGGCTGTACGTC</td>
<td>AGACGGGTAGTAGGGAAC</td>
</tr>
<tr>
<td>Muc5AC</td>
<td>AGGTTGACCCGGTTGTTGCT</td>
<td>CAGCAGGAGGAGGCGTTGTGATCT</td>
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</table>
Muc5B      CCTCTCTGTCCACCCACTA   TCCATGCGTGAAGTGAAGAG
CCL20      CGTCTGCTCTTCTTGTCTT   TTGACAAGTCCTCACTGGGACA

2.3.11. Biotinylation

To label E-cadherin at the cell surface following acrolein treatment, H292 cells were incubated with 100 µM EZ-Link™ NHS-Biotin for 1 hour to label all surface proteins, washed 2x with PBS then lysed with WSB. To label endocytosed E-cadherin H292 cells were labeled with 0.5 mg/mL EZ-Link™ NHS-SS-Biotin prior to acrolein treatment for 1 hour. Cells were washed 2x with PBS then treated with 100 µM acrolein for 3 hours. Following acrolein exposure cells were washed with cold PBS then incubated with 100 µM GSH on ice to cleave the biotin tag from proteins remaining at the cell surface. Cells were then lysed on ice with WSB, scraped and sonicated. After quantifying protein using a bicinchoninic acid (BCA) protein assay (Pierce), an equal amount of protein from each sample was washed 5x with 20 mM Tris pH 7.4 in an Amicon® Ultra 3k centrifugal spin column (Millipore) to remove excess biotin. Washed protein was brought to a volume of 500 µL using WSB and added to high capacity NeutrAvadin™ agarose beads (Thermo) to isolate biotinylated proteins. Beads were incubated at 4°C overnight then washed as previously described [54]. Eluted protein was mixed with 6xRSB then separated on SDS-PAGE for western blot.

2.3.12 Statistical analysis

Data was represented as the mean +/- SEM. Statistical significance was determined by t-test or ANOVA when more than two groups were present. Significance was set at a cutoff of p<0.05.
2.4. Results

2.4.1. Acute acrolein exposure reduces lung epithelial barrier function through Src activation.

Since Src is known to regulate the stability and expression of the adherens junction protein E-cadherin and cigarette smoke reduces epithelial barrier integrity we analyzed the effects of Src activation on the lung epithelium following acrolein exposure. We grew mouse tracheal epithelial cells in Transwell® cell culture plates and used two different methods to determine the effects of acrolein on lung epithelial barrier function. We measured the trans epithelial resistance (TER) of an epithelial monolayer using a volt-ohm meter and measured physical permeability of a cell monolayer using FITC-dextran. We observed a decrease in TER over time after treatment with 30 μM and 100 μM acrolein indicating a weakening of the epithelium (Fig 2.1A). After 24 hours apical cell media was replaced and supplemented with cell media containing 5 mg/mL FITC-dextran. We saw an increase in the permeability of cells treated with acrolein measured by an increase in FITC-dextran in basal media (Fig. 2.1B). When we analyzed protein by western blot we observed a reduction in the presence of E-cadherin and ZO-1 in acrolein treated cells consistent with results indicating loss of epithelial barrier integrity (Fig. 2.1C). To determine the role of Src on epithelial barrier function we inhibited Src activity using the pharmacological inhibitor PP2. Pretreatment with 10 μM PP2 completely prevented the loss of TER, permeability to FITC-dextran and loss of E-cadherin and ZO-1 following acrolein exposure, suggesting that Src activation is playing an important role in regulating epithelial barrier function (Fig 2.1).
After loss of epithelial barrier function was confirmed using *in vitro* models, we measured the effects of acrolein on lung epithelial permeability *in vivo*. Mice were injected with 25 mg/mL FITC-dextran in the tail vein then exposed to 5 ppm acrolein for 4 hours. The ratio of FITC fluorescence in the BALF normalized to the FITC present in the blood serum was used as a measure of epithelial barrier integrity. When BALF and serum was analyzed there was a modest yet significant increase in FITC fluorescence in acrolein exposed mice (Fig 2.2). Mice treated intranasally with the Src inhibitor AZD0530 before acrolein exposure showed a significant reduction in epithelial permeability compared to PBS controls demonstrating that Src inhibition prevents weakening of epithelial barrier integrity when exposed to acrolein (Fig 2.2).

### 2.4.2. Acrolein induces a redistribution of E-cadherin

We next looked to determine the effects of acrolein on E-cadherin visually using confocal microscopy. H292 cells were grown in chamber slides and transfected with non-specific (NS) siRNA or Src siRNA to silence Src expression. After treatment with acrolein for 4 hours we saw a reduction in E-cadherin fluorescence in NS-siRNA cells and no loss of E-cadherin in cells transfected with Src siRNA (Fig 2.3A). To analyze the levels of E-cadherin present after acrolein treatment we performed an experiment under similar conditions and lysed cells to analyze protein content by western blot. Interestingly, in the acrolein treated cells there was no significant reduction in E-cadherin at similar concentrations and time points compared to the levels measured by confocal microscopy (Fig 2.3B).
We hypothesized that acrolein was inducing E-cadherin endocytosis removing it from the cell membrane, resulting in reduced E-cadherin intensity at the membrane while total proteins levels remains the same. To test this hypothesis, we used two membrane impermeable probes to label E-cadherin at the cell surface and track its localization following acrolein treatment. Following acrolein treatment of H292 cells and labeling surface proteins with EZ-Link™ Sulfo-NHS-Biotin, E-cadherin was isolated from cell lysates using NeutrAvidin agarose beads. We observed a reduction in isolated E-cadherin after acrolein treatment while there was no change in E-cadherin levels in whole cell lysates indicating that E-cadherin endocytosis had occurred (Fig 2.4). Although we were able to prevent loss of E-cadherin through Src siRNA transfection as analyzed by E-cadherin immunofluorescence imaging (Fig 2.3A), we were not able to prevent loss of E-cadherin surface labeling through the use of Src siRNA (Fig 2.4). As an alternative labeling strategy, we utilized a biotin tag that could be cleaved off using a reducing agent. By labeling surface proteins and allowing for endocytosis, cleaving the label with a membrane impermeable reducing agent results in labeling of only endocytosed proteins that could then be purified by avadin chromatography. After performing this experiment and analyzing labeled proteins by western blot we did not observe any differences in E-cadherin between untreated and acrolein treated H292 cells. Since the biotin tag was cleaved from surface proteins this indicates that endocytosis is occurring in both treatment groups (Fig 2S1).
2.4.3. Chronic acrolein exposure results in loss of E-cadherin in vitro.

Previous reports indicate that cigarette smoke exposure results in the development of EMT through the activation of Src. To determine if chronic acrolein exposure resulted in features of EMT in vitro, we exposed mouse tracheal epithelial cells (MTECs) to acrolein every day for two weeks. After two weeks cells were lysed and analyzed by western blot and qRT-PCR to determine the presence of any epithelial alterations. qRT-PCR analysis shows an increasing trend of vimentin and Col1A1 in cells treated with 30 µM acrolein (Fig 2.5A). We did not observe any changes in expression of E-cadherin or TGFB1. Analysis by western blot shows a reduction of E-cadherin after chronic exposure to 30 µM acrolein (Fig 2.5B).

We next tested the effects of chronic acrolein exposure in vivo by exposing mice to 5ppm acrolein for 4 hours a day for 2 weeks. Preliminary experiments conducted in the laboratory had shown that chronic acrolein resulted in decreased of E-cadherin, increased vimentin and increased in Src phosphorylation detected by western blot. Additionally, we observed increased TGF-β1 expression and release detected by PCR and ELISA respectively (O’Brien et al, unpublished data). After mice were exposed to 5 ppm acrolein for 4 hours a day for 2 weeks a panel of genes involved in EMT and epithelial alterations was examined by qRT-PCR and western blot. We observed an increase in MMP9 and the neutrophil chemoattractant KC (Fig 2.6A) and upward trends of Muc5b and CCL20. We did not observe any differences in BALF cell count or Masson’s Trichrome (Fig 2.6B and C). Lastly, we did not observe any differences in protein levels of E-cadherin, vimentin, or pSrc by western blot (Fig 2.6D).
2.5. Discussion

Exposure to cigarette smoke is associated with an increased risk in the development of COPD and lung cancer. Increasing evidence supports the role of reactive oxygen species in these processes leading to DNA damage, increased inflammation, and altered signaling activity both activating and inhibitory through binding to redox sensitive cysteine thiols [38, 40]. Studies have demonstrated that Src activation by cigarette smoke is responsible for lung tissue destruction, a loss of E-cadherin and a reduction in epithelial barrier integrity [42]. We hypothesized that reactive aldehydes could induce Src activation leading to similar changes in the lung epithelium. Indeed, we show that acrolein reduces epithelial barrier function through activation of Src as seen in experiments using MTECs (Fig 2.1). Further we show evidence that acrolein incudes E-cadherin endocytosis as an early step in protein degradation (Fig 2.4).

Work by Zhang et al. 2012 established that cigarette smoke exposure led to the development of EMT through activation of Src [43]. While this work highlighted the importance of Src in the overall development of EMT, they did little to show a mechanism how this response was occurring. Our work looks deeper into the mechanism of how cigarette smoke leads to activation of Src, and the effects of active Src on epithelial function. We show evidence that acrolein activates Src and leads to the phosphorylation of p120, which is involved in stability of the adherens junction complex [50]. Following phosphorylation p120 dissociates from the adherens junction complex leading to complex instability and subsequent endocytosis [45]. It is likely that the reduction in barrier function we observe in MTECs is a result of p120 phosphorylation
and degradation of E-cadherin. Further, we show an increase in MMP9 and KC following chronic acrolein exposure in mice (Fig 2.6A) that is consistent with findings by Geraghty et al. 2014 showing these effects in response to chronic CS exposure [42].

The development of COPD and lung cancer is a gradual process that occurs over time. Similarly, EMT is not a rapid process and will not occur after a single dose of CS or acrolein. Although loss E-cadherin is known to induce EMT, it is too early to consider our observations EMT. In our chronic MTEC exposure model we see a loss of E-cadherin without any changes in E-cadherin gene expression. When E-cadherin is endocytosed from the cell surface, the adherens junction complex of proteins dissembles and no longer binds to β-catenin, which can act as a transcription factor in the nucleus [55]. This would suggest that E-cadherin protein loss would precede alterations in mRNA expression and loss of gene expression may occur due to secondary effects of β-catenin signaling in the Wnt pathway. We cannot assume our results are a direct observation of EMT, however they could be early signaling events that lead to lung epithelial remodeling. An important consideration to make in the interpretation of these studies is that acrolein is influencing numerous signaling pathways in the cell [9], therefore it would be naïve to think that all of the effects we observe are attributed solely to Src activation.

Together these studies demonstrate that acrolein exposure causes a weakening of epithelial barrier function and that Src plays a central role in this process. We observed what might be considered early events in epithelial remodeling in our chronic MTEC exposure model after two weeks of acrolein exposure, supporting our hypothesis that acrolein induces similar changes to the lung epithelium as chronic CS exposure, however
future studies are necessary to strengthen these claims. The effects of reactive aldehydes on the development of lung disease will continue to be an area of interest in future research helping better understand mechanisms of signaling events in response to cigarette smoke.
2.6. Figures

Figure 2.1. Inhibition of Src prevents loss of epithelial barrier function following acrolein exposure. MTECs were grown in Transwell® cell culture plates, pretreated with 10 µM of the Src inhibitor PP2 for 30 min then exposed to acrolein. Trans epithelial resistance (TER) was measured over 24 hours (A) *p<0.05 n=4. Permeability to the fluorescent molecule FITC-Dextran was measured after incubation on cells for 24 hours (B) #p<0.05 n=4. Cells were then lysed and protein content was analyzed by western blot (C).
Figure 2.2. Acute acrolein exposure decreases lung epithelial barrier function in vivo. Mice were given AZD0530 through nasal installation at a concentration of 50 nM in 50 µL. Mice were then injected with 5 mg/mL FITC-Dextran in the tail vein and exposed to 5 ppm acrolein for 4 hours. Serum and BALF was collected and analyzed for the presence of FITC fluorescence to determine epithelial permeability. Air n=2, ACR n=4.
Figure 2.3. **Src knockdown by siRNA prevents loss of E-cadherin at the cell membrane.** H292 cells were grown in chamber slides until 50% confluent then transfected with non-specific or Src siRNA. Cells were then treated with 100 µM acrolein for 3 hours and fixed for immunofluorescent imaging by confocal microscopy. Blue=DAPI, Red=E-cadherin, Bar = 100 µm (A). H292 cells were grown in 24 well plates then exposed to 100 µM acrolein for 3 hours. Cells were lysed and analyzed for E-cadherin by western blot (B).
Figure 2.4. Acrolein exposure reduces E-cadherin surface labeling. H292 cells were transfected with Src siRNA then exposed to 30 μM acrolein for 3 hours. Surface proteins were labeled with EZ-link Sulfo-NHS-Biotin then isolated with NeutrAvidin beads. Isolated protein eluents and whole lysates were analyzed by western blot.
Figure 2.5. Chronic acrolein exposure in MTECs. MTECs were grown to confluence and exposed to 3, 10 or 30 µM acrolein daily for 2 weeks. Protein and RNA was collected from cells and analyzed by qRT-PCR (A) and western blot (B) respectively. n=2.
Figure 2.6. Chronic acrolein exposure in vivo. C57BL/6 mice from Jackson Labs were exposed to 5 ppm acrolein daily for two weeks. RNA was isolated from whole lung tissue homogenates and analyzed by PRC and normalized to GAPDH (A) (Air n=6, ACR n=8) *p<0.05. BALF was spun at 1,500 rpm for 5 min to collect cells. Pellet was resuspended in 500 µL PBS and counted by ADVIA® hematology system (Siemens) (B). Mouse lungs were fixed in 4% paraformaldehyde and sectioned for histology. Lung sections were stained with Masson’s Trichrome and imaged on a bright field microscope at 4x magnification. Representative images shown (C). Protein was isolated from whole lung homogenates and analyzed by western blot (D).
Figure 2S1. Alterations in internalized E-cadherin are not detected following acrolein treatment. H292 cells were labeled with a cleavable biotin tag then exposed to 30 or 100 μM acrolein for 3 hours to allow for protein endocytosis. Biotin remaining at the membrane surface was cleaved by incubation with GSH. Endocytosed proteins were then isolated using NeutrAvidin beads and detected by western blot.
2.7 References


CHAPTER 3: ACROLEIN BINDS TO SRC AND INDUCES KINASE ACTIVATION

3.1. Abstract

Many of the adverse effects of cigarette smoke can be attributed to the presence of reactive electrophiles such as the \(\alpha,\beta\)-unsaturated aldehyde acrolein, which affects biological systems by primarily reacting with redox-sensitive cysteine residues. In the present studies we examined the effects of acrolein on lung epithelial cells and the importance of the redox-sensitive tyrosine kinase Src in these responses. Through the use of cell based models and recombinant Src in a cell-free system we were able to provide evidence supporting an aldehyde based signaling mechanism through direct acrolein adduction to redox sensitive amino acids. Acrolein exposure of H292 cells resulted in an increase in Src activation detected through phosphorylation of Y416 independent of Y527 dephosphorylation, which is consistent with a proposed non-canonical redox-based activation mechanism. Pretreatment with the Src inhibitor AZD0530 prevented Src phosphorylation and inhibited phosphorylation of downstream proteins p120 catenin and caveolin-1. Additionally, exposure of recombinant purified Src to acrolein resulted in an increase in kinase activity independent of tyrosine phosphorylation in a cell free system. Biochemical labeling approaches with aldehyde-reactive biotin tags revealed that Src is a direct target for acrolein-induced Michael adduction both in vitro and in vivo. Further studies utilizing mass spectrometry confirmed acrolein adduction to various cysteine residues including C245, C277 and C487, which have been implicated in redox-
dependent activation of Src. This work indicates that acrolein directly binds to Src and that acrolein exposure results in an increase in Src activity potentially through a mechanism involving redox sensitive cysteine residues.
3.2. Introduction

The α,β-unsaturated aldehyde acrolein is a highly reactive electrophile produced through the incomplete combustion of organic matter including CS, car exhaust, and coal burning power plants. Many of the adverse effects of CS can be attributed to reactive electrophiles such as acrolein through the generation of oxidative stress and depletion of antioxidants in the lung such as GSH [8], while past research has highlighted the importance of oxidative stress associated with CS exposure as a contributing factor in the development of COPD and lung cancer [6, 56, 57]. Although thiol based antioxidants such as GSH and NAC have been shown to block CS induced damage to the lung, suggesting an oxidative mechanism, analysis of GSH following CS exposure revealed the primary adduct to glutathione was acrolein, not oxidized GSH (GSSG), suggesting the protective role of antioxidants comes from detoxification of acrolein [8, 57]. Acrolein is classified as a soft electrophile that reacts primarily with soft nucleophiles through Michael addition to cysteine, lysine and histidine residues, and has been identified as the most abundant non-cancerous hazard present in CS [11, 58]. Acrolein’s reactivity with cysteine residues is of particular importance because many redox sensitive proteins contain cysteine residues. Previous work in the laboratory has shown that acrolein inhibits LPS-induced macrophage responses [16], inhibits NF-κB activation [59], and can activate MAPK signaling through alkylation of thioredoxin reductase 1 (TrxR1) [60].

The current studies presented here examined the effects of acrolein on its ability to activate the non-receptor tyrosine kinase Src kinase in an effort to better understand the underlying mechanism of activation upon exposure to CS. Activation of Src plays a key
role in cell adhesion, motility, and proliferation although its mechanism of activation remains unclear [31]. It’s been shown that CS exposure activates Src and leads to tissue destruction and features of epithelial to mesenchymal transition [42, 43]. Several different activation mechanisms have been proposed involving the phosphorylation and dephosphorylation of tyrosine residues, modification by electrophiles such as hydroquinone, and the oxidation of several cysteine residues on the protein [38, 39, 61]. Oxidative modification of Src and other receptor tyrosine kinases such as EGFR has recently been highlighted as an important process mediating kinase signaling [62, 63]. The effects of CS have largely been attributed to reactive oxygen species and oxidative stress within the cell resulting in increased mucous metaplasia, disruption of epithelial junction proteins, and tissue destruction, all of which are all processes involving Src kinase activation and are associated with the development of COPD [6, 42, 64].

Since CS exposure results in the activation of Src and previous studies have shown modification of cysteine residues are involved in kinase activation, the present studies were designed to assess the ability of acrolein to activate the non-receptor tyrosine kinase Src. We hypothesized that acrolein directly activates Src by binding to redox sensitive cysteine residues promoting an open active conformation. To test our hypothesis we characterized the effects of acrolein on Src activation through the use of cell culture models and recombinant protein kinase assays. We tracked the alkylation and oxidation of Src using biotin-conjugated probes and mass spectrometry to identify specific modified cysteine residues. Our studies confirmed that acrolein binds directly to Src and leads to increased kinase activity, as measured by a kinase activity assay and by
the phosphorylation of downstream Src targets. Together, this work emphasizes the importance of reactive aldehydes as signaling molecules found in CS and also provides further insight into understanding the activation mechanism of Src. These studies provide a better understanding of the signaling events that occur upon CS exposure that contribute to the development of lung disease and could offer insight into targets for potential future therapeutics.
3.3. Methods

3.3.1. Cell Culture

Human pulmonary mucoepidermoid carcinoma NCI-H292 cells (ATCC) were maintained in RPMI-1640 (Gibco) media containing 1% Penicillin/Streptomycin and 10% FBS (Gibco). Primary mouse tracheal epithelial cells (MTECs) were isolated as previously described by Wu and Smith [53] from C57BL/6J mice (Charles River). MTECs and human bronchial epithelial (HBEs) cells were cultured in DMEM/F12 media containing 20 ng/mL cholera toxin (List Biological Laboratories; Campbell, CA), 5 µg/mL insulin (Sigma), 5 µg/mL transferrin (Sigma), 15 µg/mL bovine pituitary extract (Invitrogen), 10 ng/mL EGF (Calbiochem; San Diego, CA), 100 nM dexamethasone (Sigma), 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. All cells were incubated at 37°C and 5% CO₂.

3.3.2. Cell Treatments

To starve cells, at least 24 hours before experiments began, growth media was replaced with serum-free media without phenol red. One hour before experiments began starvation media was replaced with fresh serum free media. A working stock of acrolein (Sigma) was made in H₂O at a concentration of 10 mM. Acrolein was added directly to cell culture media for desired final concentrations of 1-100 µM. Src inhibitors were prepared in DMSO as stock solutions of 10 mM. Inhibitors PP2 and AZD0530 were diluted to final concentrations of 10 µM and 1 µM respectively in serum free media without phenol red. Inhibitors were incubated on cells for 30 min prior to experiments. Prior to lysis, cells were washed with cold PBS then lysed for 30 min on ice using
Western Solubilization Buffer (WSB) containing 1% Triton, 50mM HEPES, 250 mM NaCl, 10% Glycerol, 1.5mM MgCl₂, 1 mM PMSF, 1 mM EGTA, 2 mM Na₃VO₄, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin. Protein levels in cell lysates were quantified with a bicinchoninic acid (BCA) protein assay (Pierce).

3.3.3. Western blot

Cell lysates containing equivalent amounts of protein were separated on 10% tris-glycine SDS PAGE gels (Biorad) and transferred to a nitrocellulose membrane (Thermo). Membranes were blocked in 5% non-fat milk for 1 hour. Antibodies were supplied by Cell Signaling Technologies unless otherwise noted. Primary antibodies for E-cadherin, vimentin and ZO-1 (Invitrogen), were diluted 1:1000 in Tris-buffered saline containing 5% BSA and 0.1% Tween® 20 (TBS-T). Beta-actin antibody (Sigma) was diluted 1:10,000 in 5% BSA TBS-T. Secondary antibodies (Cell Signaling) were diluted 1:1000 in 5% milk in TBS-T and incubated on membranes for 1 hour at room temperature. Membranes were incubated for 5 min with enhanced chemiluminescent substrate (Pierce) to identify proteins by x-ray film (Kodak) or Amersham™ Imager 600 (GE Life Sciences).

3.3.4. Mouse Lung Section Immunofluorescence

After an acute acrolein exposure (5 ppm for 4 hours) as previously described in Chapter 2, mouse lungs were removed and fixed in 4% PFA before being embedded in paraffin. Embedded lung tissue was sectioned and stained with HO-1 rabbit primary antibody (1:250) and secondary goat anti-rabbit Alexa-555 conjugated IgG (1:500). Cell nuclei were stained with 10 µg/mL DAPI then imaged as described above.
3.3.5. Src activity Assay

Src activity was determined with the Antibody Beacon™ Tyrosine Kinase Assay kit (Invitrogen) using 1.5 ng recombinant Src (Millipore T413M) or immunoprecipitated Src from H292 cells diluted in 25 µL kinase buffer. Kinase assay components were added together in a 96 well plate as described in the protocol. Src was added to wells containing kinase assay components and incubated at 37°C for 2 hours. The kinase assay kit contains an antibody that binds and quenches a fluorophore that can be displaced by a phosphorylated synthetic peptide. Increased kinase activity produces more phosphorylated peptide leading to an increase in fluorescence that can be measured by a plate reader (BioTek) at Ex/Em 492/517 nm.

3.3.6. Biotinylation

To label acrolein adducts, biotin-hydrazide (Pierce) was prepared in DMSO as a 50 µM stock. Equal amounts of cell lysates were reacted with biotin-hydrazide at a final concentration of 5 µM for 2 hours at room temperature. An equal volume of 20 µM sodium cyanoborohydride was reacted with each sample on ice for 1 hour to reduce and stabilize the hydrazine bond. DCP-Bio1 (Kerafast) was added to WSB at a final concentration of 1 mM to label sulfenic acids. Lysis buffer was supplemented with 200 U/mL catalase to remove hydrogen peroxide and 10 mM  N-Ethylmaleimide to alkylate free cysteine thiols to prevent further oxidation. Cells were lysed on ice for 1 hour then scraped and sonicated. Free cysteine thiols were labeled using WSB supplemented with 100 µM EZ-Link™ Iodoacetyl-LC-Biotin (Thermo) and 200 U/mL catalase. Lysis buffer was bubbled with nitrogen on ice to remove excess oxygen. Lysis buffer was added to
cell plates in a hypoxic chamber containing nitrogen then wrapped in parafilm and incubated at 37°C for 90 min. Cells were then scraped, sonicated and centrifuged at 14,000 rpm for 5 min to remove cellular debris. Samples were stored in -20°C.

Following all biotin labeling experiments, protein was quantified using a bicinchoninic acid (BCA) protein assay (Pierce). An equal amount of protein from each sample was washed 5x with 20 mM Tris pH 7.4 using Amicon® Ultra 3k centrifugal spin columns (Millipore) to remove excess biotin. Protein was brought to a volume of 500 µL using WSB and added to high capacity NeutrAvadin agarose beads (Thermo) to isolate biotinylated proteins. Beads were incubated at 4°C overnight then washed as previously described [54]. NeutrAvadin beads were boiled for 10 min in elution buffer containing 2% SDS, 1 mM EDTA, and 50 mM Tris-HCl at pH 8.0. Eluted protein was mixed with 6xRSB and separated on SDS-PAGE for western blot.

3.3.7. Mass Spectrometry

Recombinant Src (Millipore T431M) was exposed to a 50 molar excess acrolein for 30 min. Samples were mixed with reducing sample buffer, run on SDS-PAGE then stained with Coomassie blue. Bands containing Src were removed and de-stained using 25 mM NH₄HCO₃ in 50% acetonitrile. Gel pieces were dehydrated with 100% acetonitrile then dried using a speed vac. To reduce any disulfide bonds gel pieces were rehydrated with 10 mM dithiotreitol (DTT) in 100 mM NH₄HCO₃ for 1 hour at 57°C. To alkylate free cysteine thiols samples were incubated with 55 mM (10 mg/mL) iodoacetamide in 100 mM NH₄HCO₃ for 45 min in the dark. Gel pieces were then dehydrated with 100% acetonitrile and rehydrated with 100 mM NH₄HCO₃. After
dehydrating again with 100% acetonitrile gel pieces were dried using a speed vac. An in-gel trypsin digestion was performed using 12 ng/µL trypsin in buffer containing 5% acetonitrile, 40 mM NH₄HCO₃ in H₂O at 37°C for 12-18 hours. Peptides were extracted at room temperature by incubating gel pieces with 5% formic acid in H₂O, 5% formic acid in 50:50 H₂O:acetonitrile and 100% acetonitrile. Peptide solutions were then dried in a speed vac and analyzed using a Thermo-Fisher Scientific LTQ Linear Quadrupole Ion Trap-Orbitrap Mass Spectrometer Plus Liquid Chromatography, within the Vermont Genetics Network core facility at The University of Vermont. Peptides were identified using the SEQUEST database.

3.3.8. Statistical Analysis

To determine statistical significance data was analyzed by t-test or ANOVA based on the number of experimental groups. A p-value <0.05 was used as a cutoff of significance. Data was graphed as mean +/- SEM.
3.4. Results

3.4.1 Acrolein activates Src.

The canonical activation mechanism of Src involves the dephosphorylation of Y527 along with auto-phosphorylation at Y416 [65]. Since there is evidence that that Src can be activated through a non-canonical redox based mechanism after exposure to TGF-β and 4-hydroxynonenal, we first tested if Src activation could occur following exposure to acrolein [39, 41]. H292 cells were exposed to varying concentrations of acrolein for 90 min then lysed for analysis by western blot. We observed an increase in Y416 phosphorylation only at 100 µM (Fig. 3.1A). One concern that can arise using higher concentrations of acrolein is cell toxicity, therefore we utilized the CellTiter-Glo® luminescence cell viability assay to measure acrolein toxicity over time. At 4 hours there was a slight decrease in cell viability at 200 µM and a significant decrease at 400 µM (Fig. 3S1) confirming that the doses used for experimentation were sub-toxic.

To track activation over time, H292 cells were exposed to a bolus of 100 µM acrolein at various times from 15-120 min, and then lysed for analysis by western blot. Acrolein exposure resulted in an increase in Y416 phosphorylation beginning around 60 min and was persistent through 120 min (Fig. 3.1B). Similar to activation by TGF-β and 4-hydroxynonenal [41], acrolein exposure did not result in decreased phosphorylation of Y527 (Fig. 3.1B). Previous work in the van der Vliet laboratory has shown that 10 min exposure to ATP (100 µM) or EGF (10 ng/mL) results in increased Src phosphorylation and activation [66] and others have shown an increase in Src phosphorylation in response to 10% cigarette smoke extract (CSE) at 15 min [43]. Interestingly, we did not observe an
increase in Y416 phosphorylation at early time points (15-30 min, Fig 3.1B), which would suggest an alternative activation mechanism. We repeated time course experiments in immortalized human bronchial epithelial (HBE) cells and observed a similar pattern of activation as H292 cells, confirming previously described results were not cell type-specific (Fig. 3.1C).

We then measured phosphorylation of the downstream Src targets p120 catenin and Caveolin-1 to confirm the increase in Src phosphorylation corresponded with an increase in kinase activity. Phosphorylation of both caveolin-1 and p120 increased at the same time as Src Y416 phosphorylation, supporting our findings that Src is active (Fig. 3.1B). To confirm that these two downstream phosphorylation targets were due to Src activation, we used AZD0530 (APExBIO) to inhibit Src kinase activity. By pretreating cells with AZD0530 we were able to prevent acrolein-induced phosphorylation of Y416 on Src as well as the downstream phosphorylation of caveolin-1 and p120 detected through western blot (Fig. 3.1D). Additionally, we show that acrolein induces EGFR phosphorylation at Y1068 that can be mediated through Src inhibition (Fig. 3.1D).

We wanted to confirm that Src activation was not occurring through the activation of the EGF receptor since both Src and EGFR are known to activate each other resulting in a possible feed forward activation mechanism [66, 67]. Using the EGFR inhibitor AG1478 we were able to suppress the phosphorylation of tyrosine Y1068 following acrolein exposure (Fig 3.2). Looking at the activation of Src in the same experiment we see do not see any change in Y416 phosphorylation suggesting that EGFR activity is not involved in Src activation in response to acrolein (Fig 3.2).
Antioxidant response element (ARE) genes are induced in the cell as an adaptive cytoprotective response to oxidants and electrophilic stress. One ARE gene, heme oxygenase-1 is induced in response to acrolein and can be used as a measure of a positive acrolein exposure [68]. It has also been shown that HO-1 is induced in response to CS through activation of Src, therefore, we looked to determine if acrolein could induce HO-1 through a Src dependent mechanism [69]. HO-1 induction was confirmed in our in vivo exposure model where we observed an increase in HO-1 mRNA, protein, and immunofluorescent staining in the lung epithelium after an acute exposure of 5 ppm acrolein for 4 hours (Fig 3.3A-C). When we silenced Src expression using siRNA we see attenuation of the HO-1 response to acrolein measured by western blot suggesting that acrolein is inducing HO-1 expression through a Src dependent mechanism (Fig 3.4).

After demonstrating that acrolein could induce phosphorylation of Src targets we looked to determine if we could observe an increase in Src activity directly using an in vitro kinase activity assay with Src immunoprecipitated from H292 cells. H292 cells were exposed to 30 or 100 µM acrolein for 90 minutes then lysed in WSB. Src was immunoprecipitated then analyzed for kinase activity using the Antibody Beacon™ Tyrosine Kinase Assay Kit or analyzed by western blot for Y416 phosphorylation (Fig. 3.5A and Fig. 3.5B). We found that acrolein exposure resulted in an increase in kinase activity along with an increase in Y416 phosphorylation showing direct evidence that acrolein is activating Src in a cellular context.

Next we wanted to confirm activation directly using isolated recombinant Src in the same in vitro kinase activity assay. This allowed us to assess the effects of acrolein
directly on Src activity without the influence of any other cellular components. We exposed 1.5 ng Src to increasing concentrations of acrolein over time and assessed kinase activity using the Antibody Beacon™ Tyrosine Kinase Assay Kit (Thermo). We observed an increase in kinase activity at concentrations beginning at 30 µM increasing up to 200 µM suggesting that acrolein can activate Src directly (Fig. 3.5C). After exposing Src to acrolein the samples were mixed with 6X reducing sample buffer and analyzed by western blot to test if the increase in activation corresponded to an increase in phosphorylation at Y416. Despite being a cell free system we expected to see an increase in phosphorylation at Y416 due to autophosphorylation and excess ATP in the reaction buffer, however we did not observe any change in phosphorylation following acrolein exposure (Fig. 3.5D). This shows that acrolein is able to increase Src kinase activity independent of phosphorylation and further supports the need for a kinase assay to determine Src activity without relying on the presence of downstream phosphorylation targets.

3.4.2. Acrolein binds directly to Src

Acrolein is capable of binding to cysteine residues on redox sensitive proteins and therefore could play a role as a signaling molecule. Src contains a number of redox sensitive cysteine residues, therefore we hypothesized that acrolein is activating Src by directly binding to cysteine residues, forcing an open active conformation. We utilized a number of biochemical labeling approaches to show the presence of acrolein adducts on Src. To first identify acrolein adducts we utilized EZ-Link™ Hydrazide-Biotin (Thermo) to label protein carbonyls. When acrolein forms Michael adducts on cysteine residues it
increases the number of protein carbonyls that can be labeled with Hydrazide-Biotin. H292 cells were exposed to 100 μM acrolein for 30 min then labeled with Hydrazide-Biotin to tag all acrolein adducts. After isolating proteins using high capacity NeutrAvadin agarose beads we probed for the presence of Src by western blot and observed an increase in Src in the acrolein-treated samples indicating acrolein adduction had occurred (Fig. 3.6A). Using a complementary approach, after we derivatized cell lysates with Hydrazide-Biotin we immunoprecipitated Src using an anti Src IgG antibody. After isolating Src we probed for the presence of biotin using a streptavidin HRP-conjugated antibody to measure BH-derivatized acrolein Michael adducts. In cells treated with acrolein we observed an increase in streptavidin, which is a measure of acrolein Michael adducts (Fig. 3.6B). After we identified acrolein adducts in our cell culture model we tested if we could identify acrolein adducts in vivo. We exposed C57BL6 mice to 7.5 ppm acrolein for 4 hours then isolated lung tissues. We made lung tissue homogenates and labeled acrolein Michael adducts using Hydrazide-Biotin. Following protein derivatization and purification using NeutrAvadin beads, analysis by western blot revealed an increase in Src after acrolein treatment (Fig. 3.6C). This indicates that acrolein is forming Michael adducts with Src in vivo.

Because we observe acrolein-induced activation, based on Y416 phosphorylation of Src in H292 cells after 60 min (Fig 3.1B), we wanted determined whether the changes in Y416 phosphorylation corresponded to a change in the amount of acrolein adducts over time. Using the same Hydrazide-Biotin labeling technique as described above we exposed H292 cells to 100 μM acrolein for 30, 60, or 90 min to track the presence of
acrolein adducts over time. There was a significant increase in Src-acrolein adduct at 30 min followed by a decrease by 90 minutes, suggesting a loss of acrolein adducts, or secondary reactions of acrolein adducts (Fig. 3.7A). To determine if the loss of acrolein adducts was associated with recovery of free cysteine thiols, we performed the same time course experiment using Iodoacetyl-LC-Biotin, which labels reduced cysteine residues. As expected, avadin-purified proteins from Iodoacetyl-LC-Biotin labeled cell lysates contained a strong band corresponding to Src in untreated control cells that was reduced after 30 min of acrolein exposure (Fig 3.7B). Reduction in band intensity persisted for 90 min, indicating that the observed loss of Src-BH adducts was not due to recovery of free cysteine thiols (Fig. 3.7B). One possible explanation for a lack of free thiol recovery despite loss of acrolein adducts could be the oxidation of cysteine to a sulfenic acid. A sulfenic acid would block binding of Biotin-Hydrazide or Iodoacetyl-LC-Biotin. We utilized the probe DCP-Bio1 (Kerafast), which contains a biotin tag conjugated to dimedone to label sulfenic acids over time following treatment with acrolein. We observe a low level of sulfenylation in control cells that is increased by 30 min, and then slightly decreases by 90 minutes (Fig. 3.7C). Together these results show that acrolein is causing a modification of cysteine residues over time that are reversible and could play a role in kinase activation.

The biotinylation analyses described above are able to give us qualitative information about what modifications might be occurring, but they are not able to identify specific amino acid residues that are being tagged. In order to determine the particular amino acids of interest we turn to tandem mass spectrometry (MS-MS) to give
us information on what modifications are present following acrolein exposures. Acrolein
adduction to cysteine causes a 56.02621 Da mass increase that is unique from alkylation
by other chemicals such as iodoacetamide (57.02146 Da) or N-Ethylmaleimide (125.0477
Da). Previous work in the van der Vliet laboratory identified 769 proteins by mass
spectrometry after isolating acrolein-exposed proteins with biotin hydrazide [20]. In order
to identify acrolein adducts we first exposed recombinant Src to acrolein in a cell free
system. Following an in gel digestion with trypsin we analyzed peptides for the presence
of acrolein adducts using a (QC orbitrap) mass spectrometer. We observed about 80%
protein coverage by LC-MS/MS and saw adducts present on cysteine residues 245, 277,
400, 483 and 487 (Fig 3.8), supporting our results from western blot. Representative mass
spectra of modified and unmodified C277 are shown in supplemental Fig 3S2. We then
attempted to identify acrolein adducts on Src that was immunoprecipitated from H292
cells. Following acrolein exposure, immunoprecipitation, and digestion with trypsin we
were able identify a few peptides of Src, however the coverage was not sufficient to
detect any acrolein adducts (Data not shown). After acrolein forms Michael adducts on
cysteine residues its carbonyl can form a Schiff base with the terminal amine of lysine,
cross linking the residues. Resolution of cross-links results in the formation of a unique
lysine adduct and regeneration of free cysteine thiol. Using LC-MS/MS we were able to
detect acrolein-lysine adducts in Src following acrolein exposure suggesting lysine
residues 423, 427, 295 and 298 were involved in an acrolein cross-link to cysteine (Fig
3.8).
3.5. Discussion

Src kinase activation results in increased cellular proliferation, migration, and invasiveness through EGFR/MAPK signaling, increased turnover of focal adhesions, and increased matrix degradation [31]. As a result, increased Src kinase activity is commonly observed in cancer cells with increasing evidence of Src activation in COPD [30, 42]. The exact cause of Src activation remains uncertain since Src is a non-receptor tyrosine kinase and does not have a specific activating ligand. Recent research investigating this gap in knowledge suggests that activation occurs through the oxidation of cysteine residues [38, 70]. We hypothesized that acrolein activates Src by binding to cysteine residues thereby promoting an active state.

In our experiments with acrolein we show that activation of Src occurs without dephosphorylation at Y527 (Fig 3.1B), consistent with other studies showing activation of Src by TGFβ1 through a redox dependent mechanism [39]. Increases in kinase activity were measured by detection of phosphorylated downstream target proteins and directly through a kinase activity assay (Fig. 3.1B, Fig 3.5B). Further, we show that acrolein binds to cysteine residues C245, C277, C483 and C487, which have been shown to be oxidized upon activation suggesting that modification of cysteine residues is a contributing factor to kinase activation (Fig. 3.8).

The observed increase in kinase activity using recombinant protein were modest compared to differences observed by western blot, however directly comparing these measurements is difficult because in our recombinant system we used a synthetic peptide as the phosphorylation substrate and this is likely to have a different affinity than a Src
target such as p120 or caveolin-1. Second, in a cellular context SH2 and SH3 domain interactions with docking proteins may have an impact on kinase activity and substrate availability that is not present in our recombinant system.

Interestingly, there is opposing evidence that cysteine oxidation can inhibit Src activity. It was suggested that oxidation of C277 results in an intermolecular disulfide bond forming a Src dimer that inhibits kinase activity [40]. When we expose cells to acrolein, cysteine residues are alkylated (Fig. 3.6) which would prevent oxidation of these residues and subsequent disulfide bond formation. We observe that biotin hydrazide labeling is reversed in vitro, and in our in vivo mouse model we observe a reduction in acrolein adducts over time if we wait to sacrifice mice 1, 4 or 24 hours after exposure to acrolein (O’Brien et al. unpublished data not shown). Using DCP-Bio1 labeling we show that acrolein exposure increases the oxidation of cysteine residues (Fig 3.7C). It is possible that Hydrazide-Biotin is labeling sulfenic acids as well, therefore we pretreated H292 cells with dimedone to block non-specific labeling of Hydrazide-Biotin. We observed the same change in alkylation following NeutrAvadin pull down when cells were pretreated with dimedone suggesting any oxidative modification by Hydrazide-Biotin is negligible (Fig 3S2).

The dephosphorylation of Y527 is known to occur through the activity of protein tyrosine phosphatases such as protein tyrosine phosphatase1-B (PTP1B) and SHP-2 that are known to redox sensitive [71]. The mechanism by which PTP1B dephosphorylates tyrosine residues utilizes a reactive cysteine residue that catalyzes the transfer of the phosphate to water. It’s been shown that acrolein inhibits the activity of PTP1B through
the alkylation of the cysteine residue involved in the phosphatase reaction mechanism [35]. Consistent with those findings, after labeling proteins with Hydrazide-Biotin and isolating with NeutrAvadin, we detect PTP1B by western blot confirming acrolein adduction (Fig. 3S3). The inhibition of PTP1B and other phosphatases can explain why we do not observe a decrease in Y527 phosphorylation after acrolein exposure (Fig 3.1B). Additionally, the phosphatase known to remove pY416 (PTP-BAS) could be inhibited, which could lead to enhanced levels of phosphorylation [33].

Of the nine cysteine residues found throughout Src, residue 277 is of particular interest because of its involvement in kinase activation and its close proximity to the A-loop that contains Y416. Kinase activation by cysteine oxidation or alkylation could be explained by an increase in steric hindrance, forcing the A-loop into a conformation more permissive of phosphorylation. Under basal conditions it is predicted that there is a 3.5 Å distance between cysteine and tyrosine that is increased to 4.5 Å following acrolein adduction that could force the A-loop open (Fig 3.9). Giannoni et al. 2014 speculate that Src oxidation and enhanced kinase activation can be attributed to an intramolecular disulfide bond [70], however they do not provide direct evidence of an intermolecular disulfide. Since acrolein would prevent disulfide bond formation by adducting to cysteine residues, this would suggest that the activation we observe following acrolein exposure is not due to a disulfide bond, but due to the presence of a cysteine modification or adduct. Additional mass spectrometry analysis was performed identifying the remnants of cysteine-lysine cross-links (Fig 3.8). We were specifically interested in lysine 423 because it is located in the kinase domain in close proximity to C277 and Y416. As an
alternative to cysteine modification inducing activation as described above, we hypothesized that a cysteine-lysine cross-link would form between C277 and K423 after acrolein exposure, and the resulting lysine modification would force the A-loop open. We were able to identify +38 Da mass shifts on lysine residues including K423 suggesting cross-link formation may play a role in kinase activation (Fig. 3.8).

In the studies outlined here we show that acrolein exposure leads to activation of Src and the phosphorylation of p120 and caveolin 1 (Fig 3.1), which are known to play a role in maintaining proper airway epithelial junction stability. Src activation has long been established as a mediator of signaling events in cancer with increasing evidence supporting a redox based mechanism of activation. We demonstrate that acrolein directly binds to Src and results in direct kinase activation, providing evidence of a novel mechanism of Src activation (Fig 3.9). Despite there being thousands of active chemicals present in CS we show that reactive aldehydes such as acrolein may play an important role in the development of lung diseases through mediating the activation of Src in these processes. Determining the molecular modifications underlying Src activation will help us understand how Src is involved in the development of lung disease and provide a focus for the development of future therapeutics.
Figure 3.1. Acrolein increases Src phosphorylation and activation in vitro. H292 cells were exposed to varying concentrations of acrolein for 90 min then analyzed by western blot (A). H292 and HBE cells were exposed to 100 µM acrolein at time points indicated then lysed for analysis by western blot (B and C). H292 cells were pretreated with 1 µM AZD0530 for 30 min then treated with 100 µM acrolein for 90 min. Src, EGFR, p120, and caveolin-1 phosphorylation was measured by western blot (D).
Figure 3.2. Acrolein induced Src phosphorylation occurs upstream of EGFR kinase activation. H292 cells were pretreated with the EGFR inhibitor AG1478 at a concentration of 1 µM for 30 min then exposed to 30 or 100 µM acrolein for 90 min. Analysis by western blot reveals inhibition of EGFR phosphorylation without inhibition of Src phosphorylation.
**Figure 3.3. Acute acrolein exposure induces HO-1 *in vivo*.** Mice were exposed to 5 ppm acrolein for 4 hours then sacrificed. HO-1 was analyzed by qRT-PCR (A), western blot (B), and immunofluorescent staining of lung tissue sections (C). blue=DAPI, red = HO-1, bar = 100 µm. *p<0.05
Figure 3.4. Acrolein induced HO-1 expression is mediated through Src. H292 cells were transfected with non-specific (NS) or Src siRNA then exposed to acrolein for 4 hours. Cells were lysed and analyzed by western blot.
Figure 3.5. Src activity is increased after exposure to acrolein. H292 cells were exposed to 30 or 100 µM acrolein for 90 min followed by a Src immunoprecipitation. IP Src was then tested for kinase activity (A) n=2 *p<0.05, and levels of Y416 phosphorylation by western blot (B). Recombinant Src was exposed to acrolein then measured for activity using the Antibody Beacon™ Tyrosine Kinase Assay Kit (C) n=5 *p<0.05. Recombinant Src was exposed to varying concentrations of acrolein then analyzed by western blot for Y416 phosphorylation (D).
Figure 3.6. Acrolein adducts on Src can be detected in vitro and in vivo. Following exposure to acrolein, H292 cell lysates were labeled with Hydrazide-Biotin to tag acrolein adducts. Labeled proteins were isolated using NeutrAvidin agarose beads and identified by western blot (A). Conversely, Src was immunoprecipitated from biotin-labeled cell lysates using an α-Src antibody and then probed for the presence of biotin using streptavidin-HRP (B). Whole lung homogenates taken from mice exposed to 7.5 ppm acrolein for 4 hours were labeled with biotin hydrazide, purified with NeutrAvidin agarose and analyzed by western blot (C).
Figure 3.7. Cysteine modifications by acrolein are reversed over time. H292 cells were exposed to 100 µM acrolein for various times over 90 min. Proteins in cell lysates were labeled with Hydrazide-Biotin (A), DCP-Bio1 (B) and Iodoacetyl-LC-biotin (C) to label and isolate carbonyls, sulfenic acids and free thiols respectively. Labeled proteins were isolated using NeutrAvidin™ Agarose beads and compared to whole lysates by western blot.
Figure 3.8. Acrolein adducts are detectable by mass spectrometry. Recombinant Src protein was exposed to a 50 molar excess of acrolein, run on a reducing SDS-page gel and stained with Coomassie blue. An in gel digested was performed with trypsin then samples were subjected to LC-MS/MS. Acrolein was observed on the cysteine residues listed. Observed mass (MH+), charge (+Z), and cross correlation score are given for each peptide. Asterisk (*) next to residue number indicates a residue involved in redox activation of Src activity.
Figure 3.9. Proposed Src activation mechanism. Under basal conditions Src is held in a closed inactive conformation. Upon activation, Src unfolds and allows for autophosphorylation at Y416. We propose that acrolein binds to C277 forcing the activation loop open through steric interactions between Y416 and K423, resulting in a conformation that promotes phosphorylation and activation.
Figure 3S1. Cell viability after acrolein exposure. H292 cells were exposed to acrolein for 4 hours. The effects of acrolein on cell viability was analyzed by CellTiter-Glo® luminescence cell viability assay. n=8 *p<0.05
Figure 3S2. Representative LC-MS/MS spectra. Recombinant Src was exposed to a 50 molar excess acrolein and analyzed by LC-MS/MS with a mass tolerance of 0.02 Da. Peptide spectrum (A) is shown with a parent ion mass of 2155.96 Da while peptide spectrum (B) is from a peptide mass of 2154.97 Da. The 1 Da mass shift is due to acrolein.
Figure 3S2. Dimedone pretreatment does not reduce Hydrazide-Biotin labeling.

H292 cells were incubated with 1 mM dimedone for 1 hour. Cells were then exposed to acrolein and labeled with Hydrazide-Biotin. After proteins were isolated with NeutrAvidin beads EGFR and Src were identified by western blot.
**Figure 3S3. Acrolein adducts to PTP1B.** H292 cells were exposed to 100 μM acrolein for 30, 60, or 90 min. Proteins were labeled with Hydrazide-Biotin and isolated with NeutrAvidin agarose beads. PTP1B was identified in lysates and eluents by western blot.
3.7 References


CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

4.1 Summary

Src is involved in regulating E-cadherin and has been shown to mediate the development of EMT in response to cigarette smoke [43]. Loss of E-cadherin is associated with EMT, and increasing evidence shows EMT is involved in the development of COPD as well [72]. We tested the effects of acrolein on epithelial barrier function using mouse tracheal epithelial cells seeded in Transwell® cell culture dishes measuring trans epithelial resistance and permeability to FITC-Dextran. As expected we show that acrolein exposure results in a weakening of the epithelium (Fig. 2.1). We demonstrate that Src activation is a key mediator in this response since Src inhibition with the inhibitor PP2 could completely prevent loss of epithelial barrier integrity (Fig 2.1). Further, we show that loss of barrier function corresponds to a subsequent loss of epithelial junction proteins E-cadherin and ZO-1, and the loss of protein could be attenuated through Src inhibition (Fig 2.1C).

It’s been shown that cigarette smoke exposure leads to the development of EMT and lung tissue destruction through activation of Src [42, 43]. Preliminary experiments have shown that chronic acrolein exposure leads to a reduction in E-cadherin (Fig. 2.5), which is a hallmark of EMT and associated with the development of COPD. In our in vivo chronic exposure models we were not able to replicate preliminary findings that acrolein exposure resulted in decreased E-cadherin and increased vimentin expression in association with increased Src phosphorylation. Despite these findings we show that
acrolein increases MMP9 and KC expression (Fig 2.6) and that acrolein induces an HO1 response (Fig 3.3). It should be noted that the exposure system had been slightly modified between preliminary work and these attempts at replication due to a change in facilities, although measures were taken to recreate the model to the best of our abilities. Future experiments will be required to confirm current and past findings.

We began these studies characterizing the ability of acrolein to activate the non-receptor tyrosine kinase Src in lung epithelial cells. We exposed cells to acrolein and observed an increase in autophosphorylation of Src as well as an increase in phosphorylation of downstream Src targets confirming Src was active (Fig 3.1). Interestingly Src activity increased without a reduction in the inhibitory phosphate site of Y527 (Fig 3.1B), which is consistent with a mechanism observed in redox activation of kinase activity [38, 41]. Using the chemical Src inhibitor AZD0530 we showed that downstream phosphorylation could be blocked confirming phosphorylation was mediated through Src (Fig 3.1D). We wanted to confirm that Src was the direct target of activation by acrolein, so used recombinant Src to show that activity increases in response to acrolein in a cell free system (Fig 3.5C).

Research has shown evidence that cysteine oxidation is involved in activation of Src activity [38]. Based on these findings and the chemistry involved between acrolein and cysteine residues, we investigated the ability of acrolein to bind to Src and further identify which specific residues were being adducted. Using biochemical-labeling strategies involving Hydrazide-Biotin and NeutrAvadin purification we demonstrated that acrolein binds to Src both in vitro and in vivo (Fig 3.6). We used tandem mass
spectrometry to determine which specific residues were adducted by acrolein (Fig. 3.8). We show that acrolein binds to cysteine residues that have previously been implicated in the oxidative activation of Src supporting our hypothesis that acrolein activates Src through a direct modification of cysteine residues. Studies outlined thus far were designed to determine the ability of acrolein to activate Src and provide evidence of a potential mechanism by which acrolein causes Src activation.

Together our results show that acrolein activates the Src kinase likely through direct modification of redox-sensitive cysteine residues. This demonstrates a novel activation mechanism by which acrolein adducts force the kinase domain open allowing for autophosphorylation and activation independent of the inhibitory actions of Y527. Additionally we show that acrolein induced epithelial injury and remodeling occurs through activation of Src, further demonstrating the involvement of Src activation in the development of lung disease. This work has important implications characterizing acrolein alongside ROS as a contributing factor in Src activation providing valuable information that may be useful in developing novel future therapeutics.
4.2 Future Directions

We have shown that acrolein activates Src and binds to specific cysteine residues that have been implicated in the activation of kinase activity. We have also shown that acute acrolein exposure induces epithelial barrier disruption \textit{in vitro} and \textit{in vivo} mediated through the activation of Src. Further, chronic acrolein exposure \textit{in vitro} resulted in the loss of E-cadherin, which is similarly observed in response to CS. Experiments using chemical Src inhibitors such as PP2 or AZD0530 in conjunction with acrolein exposure \textit{in vivo} will need to be repeated to provide more replicates and to ensure changes in FITC leakage are consistent. Follow up studies involving chronic acrolein exposure will also be conducted using a Src deficient mouse model to test the importance of Src activation in responses generated by acrolein exposure.

In our studies we determined that acrolein activates Src and directly binds to cysteine residues C245, C277, C400 and C487, which are involved in and oxidative activation mechanism [38]. In future studies we plan to expand upon this work by utilizing cysteine to alanine mutants to determine which specific residues are involved in increasing kinase activity in response to acrolein. Studies will be conducted using recombinant Src mutants purified from \textit{E.coli} and in H292 cells transfected with plasmids containing different Src mutants. We can perform further experiments with these mutants addressing aspects of cell migration or wound healing in response to acrolein to assess the role of Src activation in a more physiologically relevant context. Identifying the specific residues involved in activation will help determine a mechanism describing Src
activation and can provide insight into what interactions are occurring within Src that lead to kinase activation.

Current mass spectrometry experiments have identified cysteine residues as direct targets for acrolein adduction, although experiments thus far have identified adducts only at single time points. Additionally, using our current models we cannot determine the degree of alkylation within a sample, or quantitative changes in alkylation over time. We would like take advantage of isotopic labeling strategies to quantify acrolein adducts over time using mass spectrometry and to see if increases in Src phosphorylation can be correlated to the rise or fall of acrolein adducts on specific cysteine residues. These experiments can be conducted using commercially available reagents with little to no alteration in initial experimental procedures.

We first observed an increase in Src oxidation in experiments using the dimedone based biotin tag DCP-Bio1 (Fig. 3.7C). It was not too surprising to see a slight rise in oxidation following acrolein exposure since acrolein binds and depletes many cellular antioxidants, specifically GSH [23]. With a loss of cellular antioxidants we would expect to see a rise in the levels of ROS within the cell creating a more oxidizing environment. Since Src is activated in response to reactive oxygen species that oxidize cysteine residues we plan on investigating the involvement of ROS on Src activation following acrolein exposure. Initial experiments will utilize superoxide indicators such as hydroethidine or dichlorofluorescein (DCF) that can track ROS production in plate reader assays or confocal microscopy. ROS scavengers such as catalase or NAC will be used to characterize the impact of ROS on Src activation in response to acrolein. Further, we can
use mass spectrometry to identify oxidized cysteine residues on Src following immunoprecipitation from cell lysates after acrolein exposure. Research investigating the interplay between alkylation and oxidation will help determine mechanisms involved in protein activation. In the future we hope to expand upon the current completed studies to better understand the effects of acrolein on the lung epithelium and the role of Src in these responses.


32. Boerner, R.J., et al., Correlation of the phosphorylation states of pp60c-src with tyrosine kinase activity: the intramolecular pY530-SH2 complex retains


